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(54) Title: METHOD FOR DETECTION OF HUMAN PAPILLOMAVIRUS (HPV) FOR DIAGNOSTIC PURPOSES

(57) Abstract

Method for detection of infection with human papillomavirus (HPV) for diagnostic purposes, particularly for diagnosing carcinoma, or pre-stages thereof, or the risk of development of carcinoma. The detection is effected on a body fluid, particularly n a secretion from cervix uteri, by ascertaining the presence of IgA, IgG and IgM antibodies against papillomavirus virions, including individual virion proteins or peptides thereof, in the body fluid.

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METHOD FOR DETECTION OF HUMAN PAPILLOMAVIRUS (HPV) FOR DIAGNOSTIC PURPOSES

The invention relates to a method for detection of infection with human papillomavirus (HPV) for diagnostic purposes.

Human papillomavirus (HPV) infection of the cervix uteri is associated with carcinoma of the cervix uteri. Over 50 different HPV types have been identified. HPV types 6, 11, 16, 18, 31, 33 and 51 have been found to infect the genital tract. Types 6 and 11 cause benign proliferative lesions in the genital tract, condylomata acuminata. Types 16, 18, 31 and 33 are found in pre-cancerous lesions and in a majority of carcinomas of the cervix uteri. The human papillomaviruses are immunologically related to the bovine papillomaviruses: antisera reactive with both groups of viruses can readily be prepared. Papillomavirus capsid antigens have been demonstrated in pre-cancerous lesions and condylomatous tissue using such group-specific antisera prepared against viral capsids. Patients with genital warts, cervical intraepithelial neoplasia (CIN) and carcinoma of the cervix uteri have been reported to have higher serum IgG antibody levels to the group-specific capsid antigen compared to control groups.

The object of the invention is to provide a method of the type referred to above in order to facilitate the detection of carcinoma, particularly cervical carcinoma, or pre-stages thereof, or the risk of development of carcinoma.

Another object of the invention is to provide a method of the type referred to above by means of

which the presence of carcinoma, or pre-stages thereof, or the risk of development of carcinoma, can be ascertained in a simple and rapid way by detection of the presence of antibodies to human papillomavirus (HPV) in body fluids.

In order to achieve these and other purposes which will be apparent from the description which follows the method of the invention has obtained the characterising features of claims 1, 11 21 and 31.

The invention will be described in more detail below, reference being made to the accompanying drawings in which

FIG. 1 is a graph illustrating the detection of IgA antibodies against papillomavirus in cervical secretions,

FIG. 2 is a graph illustrating the correlation of IgA antibodies to papillomavirus in serums and cervical secretions,

FIG. 3 is a graph illustrating the comparison of IgG antibodies to papillomavirus in serum and vaginal secretions,

FIG. 4 is a graph illustrating the comparison of IgA and IgG antibodies against papillomavirus in vaginal secretions,

FIG. 5 and 6 are graphs illustrating the detection of IgA and IgG antibodies by immunoblotting,

FIG. 7 is a graph illustrating age and sex-related distribution of serum IgA antibodies to PV in a normal population,

FIG. 8 is a graph illustrating age and sex-related distribution of serum IgG antibodies to PV in a normal population,

FIG. 9 is a graph illustrating detection of IgA antibodies against PV in serum from patients with CIN or carcinoma of the cervix uteri,

FIG. 10 is a graph illustrating detection of IgG antibodies to PV in serum from patients with CIN or carcinoma of the servix uteri,

FIG. 11 is a graph illustrating detection of IgM antibodies to PV in serum from patients with CIN or carcinoma of the servix uteri,

FIG. 12 is a graph illustrating the difference between the IgA and IgG response to PV in relation to disease,

FIG. 13 is a graph illustrating IgA reactivity to the L1 protein,

FIG. 14 is a graph illustrating IgA reactivity to the L2 protein,

FIG. 15 is a graph illustrating IgA reactivity to the L1 protein,

FIG. 16 is a graph illustrating IgG reactivity to the L2 protein,

FIG. 17 is a graph illustrating IgM reactivity lto the L1 protein,

FIG. 18 is a graph illustrating IgM reactivity to the L2 protein, and

FIG. 19 is a graph illustrating disease-associated reactivity of synthetic peptides.

Table 1A is a graph tabulating comparisons between IgA and IgG antibodies to PV in serum and cervical secretions.

Table 1 is a graph illustrating detection of cervical carcinoma-associated antibodies.

Table 2 is a graph explaining the formula used for synthetic peptides.

Table 3 is a graph illustrating the amino acid sequences of the synthetic peptides.

In order to investigate if IgA antibodies against papillomavirus exist and whether they are correlated with progression to malignancy, cervico-

vaginal secretions were examined from patients with condylomata and CIN using a modified ELISA technique.

Fortytwo women 20-50 years old participated in the study. They had a previous abnormal vaginal smear and/or condylomas or participated in a screening program. All patients underwent colposcopic examination. A regular Papanicolau smear was taken at the day of colposcopy and in some cases biopsies were taken from colposcopically verified lesions. Cytological and histological examinations were made and matched to ELISA results at the end of the study. Morphologic criteria of HPV infection were made according to Meisels et al (1979). Active papillomavirus infection was suggested by the finding of koilocytotic cells. The typical koilocytotic cell is an intermediate cell with enlarged, hyperchromatic nucleus surrounded by a clear cytoplasmic zone. Cervico-vaginal secretions were collected from endo-ecto cervix and vagina with a small swab. The swab was placed in a glass tube filled with 0.5 ml PBS and mixed on a Vortex mixer. The specimens were stored at -20° C until used. The exact amount of secretion was estimated by weighing the tube before and after collection. Before use they were centrifuged to remove debris. Samples were collected regardless of day in the menstrual cycle excluding only actual days of menstruation. Blood-stained specimens were discarded.

BPV had been purified as follows: Bovine cutaneous warts stored in glycerolphosphate-buffered saline (1:1) at $+4^{\circ}$C were homogenized in 0.1 M Tris-HCl, pH 7.5 in an ultra turrax mixer. A 10 % suspension was prepared and, after addition of tareosyl and freon, the suspension was centrifuged at 11.950 g for 30 min in a Sorvall SS-34 rotor. The

resulting supernatant was centrifuged at 35,000 rpm for 60 min in an MSE 8x40 ml titanium rotor. The pellet was suspended in 0.1 M Tris-HCl and mixed with CsCl. The solution was centrifuged to equilibrium at 35,000 rpm in an MSE SW50 rotor overnight. The virus bands were collected, dialyzed against TE buffer (0.01 M Tris-HCl, 0.001 M EDTA) and stored frozen at -70°C. Extracts of cutaneous warts were purified by 2 cycles of equilibrium centrifugation in CsCl. Two distinct bands were seen in the gradients. The lighter band had a bouyant density of 1.29 g/ml; it consisted of empty virus particles as determined by electron microscopy (EM). The heavy band had a bouyant density of 1.34 g/ml and contained complete virus particles with a typical papovavirus morphology also as determined by EM.

Hyper-immune sera against purified bovine papillomaviruses had been prepared in rabbits by intramuscular inoculation of approximately 100 µg of purified virus, emulsified in Freund's complete adjuvant (Difco laboratories, Detroit, Mich.). Two subsequent inoculations were made two weeks apart, using the same amount of virus but without adjuvant. The rabbits were bled 1 week after the last inoculation.

A modification of previously described ELISA methods was used. Briefly, BPV virions diluted in PBS were added to a 96-well microtitration plate and kept overnight at +4 °C at a fixed concentration of 0.06 µg/well. After three washes with PBS containing 0.05 % Tween (PBS-Tween), the plates were blocked with 4 % bovine serum albumin (BSA) and 0.1 % gelatin in PBS for 6 hours at room temperature, then left overnight at +4°C. Before use, plates were washed 5 times with PBS-Tween. The cervico-

vaginal secretions were diluted 1/40 and sera were diluted 1/100 in 4 % BSA, 0.1 % gelatin and 0.05 % Tween. To detect bound antibodies, affinity-purified biotinylated anti-human IgA (alpha chain-specific) or biotinylated anti-human IgG (both from Sigma) were used at a dilution of 1/1000 for 4 hours at room temperature. Peroxidase-conjugated avidin was then added at a dilution of 1/400 for 2 hours at room temperature. As substrate 0.4 % o-phenylenediamine (Sigma), 0.012 % H_2O_2, in 50 mM phosphate-citrate buffer, pH 5.0, was used. Positive controls were rabbit antisera against BPV virions and/or rabbit antisera against elk papillomavirus. Human newborn sera and rabbit pre-immune sera were negative controls.

BPV virions were electrophoresed on a 5-20 % polyacrylamide gradient gel. About 5 μg of virions were applied to each sample lane. Transfer to nitrocellulose sheets was done as described by Towbin et al. The sheets were cut into strips and blocked with 2 % Tween-80 in 50 mM Tris pH 10.2, 150 mM NaCl, 5 mM NaN_3 for 15 minutes. The nitrocellulose strips were incubated overnight with patients sera diluted 1/100 in wash buffer (50 mM Tris pH 10.2, 150 mM NaCl, 5 mM NaN_3, 0.1 % Tween-20). After an overnight incubation the blots were washed in the same buffer. Rabbit antiserum against PV was used as positive control and rabbit pre-immune serum as negative control. For detection of bound antibodies biotinylated goat anti-human IgA or biotinylated goat anti-human IgG was used, diluted 1/1000 in wash buffer. The blots were subsequently incubated with peroxidase-conjugated avidin diluted 1/400 in wash buffer. For the rabbit antisera, goat anti-rabbit IgG conjugated to per-

oxidase was used, diluted 1/1000 in wash buffer. All blots were developed with 0.02 % carbazole (Sigma) in 50 mM sodium acetate, pH 5.5.

FIG. 1 illustrates the detection of IgA antibodies against papillomavirus.
The cervical secretions, diluted 1/40, were applied to ELISA plates coated with purified and disrupted BPV virions. IgA antibodies were detected with an IgA-specific biotinylated second antibody and avidin peroxidase conjugate. Following addition of substrate, the absorbances at 490 nm were recorded after 15 minutes. Absorbance values >0.1 above background (human newborn serum) were scored as positive.

IgA antibodies to PV were found in cervical secretions from 17 of 42 women. In the graph of FIG. 1 each point represents the mean absorbance of sample duplicates from each patient. Of 9 women with CIN, 8 had IgA antibodies in their cervical secretions against PV, group I (patients with histologically confirmed intra-epithelial neoplasia, CIN). Koilocytosis in Pap smear and colposcopically verified condylomas without CIN were found in 9 patients, and 3 of those had IgA antibodies against PV, group II (patients with koilocytosis and/or condyloma but no histological evidence of CIN). Six of 24 women with normal examination results had IgA antibodies against PV, group III (patients without any pathological findings). The IgA ELISA absorbances were tested for significant differences among the three groups. In a three-way nonparametric continuous test (Kruskal-Wallis test), the CIN group had significantly higher absorbance values than the normal control group ($p<0.015$). The proportion of IgA positive cervical secretions was also found to be significantly higher in the CIN group (8 of 9)

than in the normal group (6 of 24), ($p<0.005$, Chi^2-test). By contrast, there was no difference between the group with condylomas/koilocytosis and the normal group ($p>0.05$; absorbance mean = 0.109 versus 0.104).

For 15 patients IgG and IgA levels in serum (diluted 1/1000) and secretions (diluted 1/40), were compared. The IgA antibody levels in serum and secretions were relatively well correlated: $p<0.001$, as shown in FIG. 2. The levels of IgG antibodies in serum and secretions were not significantly correlated: $0.05<p<0.1$ as shown in FIG. 3. No significant correlation ($p>0.1$) was found between IgA and IgG levels in secretions as shown in FIG. 4, or in serum (not shown).

In order to investigate which virion polypeptides the PV antibodies were directed against, immunoblotting of the purified BPV virions was performed.

Reference is made to FIGS. 5 and 6. The purified BPV virions were electrophoresed on a 5-20 % polyacrylamide gel and transferred to nitrocellulose. Strips were incubated with rabbit hyperimmune serum against purified bovine virions (+), human newborn serum (-) and 10 patient sera (1 to 10). Bound antibodies were detected with biotinylated goat anti-human IgG (FIG. 5) or biotinylated goat anti-human IgA (FIG. 6) and peroxidase-conjugated avidin. In the IgA test, strips with patient sera No. 2-9 were equally negative as serum No. 1 and are not shown. In FIGS. 5 and 6 the arrows denote the major PV proteins detected, K = molecular weight in kilodalton, and MW = molecular weight markers. The molecular weights of the markers were 200, 116, 92, 66, 44 and 29 kilodaltons (kDa).

Rabbit hyperimmune sera prepared against the purified bovine virions detected four polypeptides: a major 14-kDa protein, 28-kDa and 54-kDa proteins, and a 64K protein, FIG. 4. Ten of ten tested patients had serum IgG antibodies to the 54-kDa protein. Two patients had serum IgG antibodies to the 64-kDa protein and 2 patients had serum IgG antibodies to the 28-kDa protein. When tested with the IgA-specific conjugate, only one out of ten patient sera had detectable IgA antibody levels in immunoblotting, FIG. 6. This serum had an exceptionally high IgA anti-BPV reactivity as determined by ELISA (compare FIG. 2). In immunoblotting it reacted with the 14-kDa, the 28-kDa and the 54-kDa polypeptides and weakly with the 64-kDa polypeptide (FIG. 6).

It has been shown that local genital tract papilloma virus antibodies exist and can be readily detected and measured. There was found a strong correlation between the presence of IgA antibodies to PV in cervical secretions and the histological diagnosis of CIN. However, 6 of 24 women with normal Pap-smear and colposcopy also had IgA antibodies against PV. It is not known if the patients with IgA antibodies but no CIN may have had histologically undetectable CIN lesions. A previous study has suggested that IgG antibodies to the papilloma virus group-specific antigen are elevated in CIN. However, serum IgG antibody levels to PV were also elevated in patients with cutaneous warts. This problem was overcome by measuring only those antibodies that are related to genital tract PV infection, by measuring the IgA antibodies described here. It should be emphasized that the herein described test was found to have no correlation to the previously described

test using IgG antibodies. This is concluded from our demonstration that there is correlation between IgA antibodies to HPV in serum and secretions (FIG. 2) but no correlation between IgA and IgG antibodies to HPV in secretions (FIG. 4) or in serum (Table 1A). There was a correlation between the levels of IgA antibodies in serum and secretions. It is therefore possible that IgA antibodies in serum may provide a genital-tract specific test that could easily be applied as part of a clinical routine.

The finding that the normal subjects and the patients with evidence of viral replication (koilocytosis and/or condyloma) had IgA antibody levels similar to those of normal subjects may seem surprising. However, this situation is also found for the Epstein-Barr virus, where anti-virus capsid IgA antibodies are not related to the level of virus production but only to the presence of virusassociated cancer. Immunoblotting detected four virion-associated polypeptides. The presence of IgG antibodies to a 54-kDa protein is in accordance with previous studies: A 56-kDa regularly IgG-immunogenic protein encoded by the L1 open reading frame has been identified as a major component of the capsid. A minor approximately 70-kDa capsid protein is encoded by the L2 open reading frame. This protein should correspond to the 64-kDa protein detected by us. The identities of the 28-kDa protein and the 14-kDa proteins are not known. The single IgA-positive serum had an IgA response to the 54-, 28- and 14-kDa polypeptides, whereas the IgG response of the same serum was only directed against the 54-kDa polypeptide. This shows that the PV epitopes which give rise to IgA and IgG are not always identical.

In order to analyse the serum antibody response to the group-specific PV capsid antigens, the prevalence of serum antibodies against PV in a normal, healthy population compared to the serum antibody titers in sera from women with CIN or cervical carcinoma was studied.

A total of 139 control sera from healthy, adult women were obtained. 62 sera were from women attending gynecology outpatient clinics. The women either had no pathological findings (48 women) or had condyloma, but no histopathological evidence of CIN (14 women). 59 sera were obtained from healthy, female laboratory workers and 18 sera from women attending an annual health check-up.

The patient group consisted of 114 women with untreated, histopathologically confirmed cervical neoplasia, of which 13 lesions were classified as CIN 1, 16 lesions were CIN 2, 16 lesions were CIN 3 and the remaining 69 lesions were invasive cervical carcinoma. 83 sera from children at various ages, as well as from adults of both sexes also were obtained.

Virus isolation and purification had been performed as described above.

The preparation of the bovine papillomavirus is shown in FIG. 5 and 6 to consist of four proteins all of which contain epitopes immunoreactive with human sera as shown by immunblotting.

Purified BPV was disrupted by five cycles of freeze-thawing and diluted in 10 mM carbonate buffer, pH 9.6 and added to half-area 96-well microtiter plates at a fixed concentration of 0.15 µg/well. The plates containing the BPV were kept at room temperature over night. After one wash with PBS-0.05 % Tween 20 (PBS-T) the plates were blocked

with 10 % lamb serum (heat inactivated; Flow) in PBS and incubated for 60 minutes at 37°C. The blocking solution was then discarded and the plates tapped thoroughly against paper. Human sera were diluted 1:20 in 10 % lamb serum/PBS, added to the plates in duplicate wells and allowed to react for 120 minutes at 37°C. The plates were then washed five times with PBS-T. To detect bound antibodies there was used a horseradish peroxidase labeled monoclonal antibody against human IgA (Janssen) (Fig. 3) diluted 1:500 in 10 % lamb serum/PBS incubated on the plates for 120 minutes at 37°C. The plates were then washed five times with PBS-T and developed with 20 mg/ml 2,2'-azino-di (3-ethylbenzthiazolin-sulfonat (6)) deammonium salt (ABTS) diluted 1:50 in 0.1 M citrate buffer, pH 4 with .9 % hydrogen peroxide. The absorbance were recorded at 415 nm after 30 minutes (FIGS. 7, 8) or 60 minutes (FIGS. 9-12). For detection of IgG, the plates were washed and blocked with 10 % lamb serum-PBS for 60 minutes at 37°C.

Then, there was used a rabbit anti-human IgG-alkaline phosphatase conjugate (Dako) diluted 1:1000 in 10 % lamb serum PBS for 120 minutes at 37°C (FIG. 9-12) or a horse radish peroxidase conjugated monoclonal antibody against human IgG (Janssen), diluted 1:2000 (FIG. 7, 8). After washing five times with PBS-T, 1 mg/ml phosphatase substrate (Sigma) in 0.1 M diethanolamine buffer, pH 9.6/1mM $MgCl_2$ was added and the plates were read at 405nm after 90 minutes. When the peroxidase-conjugated antibody was used, the plates were developed for 30 minutes as described above for the IgA conjugate. For detection of IgM antibodies, the plates were washed and blocked as described above and then in-

cubated with an anti-human IgM-glucose oxidase conjugate (Sera-lab) at dilution 1:800 in 10 % lamb serum/PBS for 120 minutes. 0.36 mg/ml of ABTS, 2.4 % glucose, 8 µg/ml horseradish peroxidase (Sigma) in 0.1 M phosphate buffer, pH 6.0, was used as substrate. The plates were read at 415 nm after 60 minutes. For all ELISAs an absorbance of 0.15 or more above background (same serum on uncoated wells) was considered a positive reaction. Two CIN patient sera with known reactivity were used as internal standards in all tests. As negative control served uncoated wells in duplicates.

The mean of duplicate absorbances, with mean of duplicate absorbances on uncoated wells subtracted,, were analysed for statistically significant differences between the patient group and the healthy control group by a two-sided, non-parametric ranking test (Mann-Whitney test).

The results were as follows:

Of 83 sera from a normal population consisting of both children and adults of both sexes, 24 had IgA antibodies and 46 had IgG antibodies against PV. The IgA anti-BPV titers were found to be elevated ($p<0.002$, Mann-Whitney test) in healthy males compared to healthy females as illustrated in Fig. 7, which shows age and sex-related distribution of serum IgA antibodies to PV in a normal population. Sera were diluted 1:20 and IgA antibodies against BPV were detected with an alpha-chain specific horseradish peroxidase conjuaged monoclonal antibody. Following addition of substrate, the absorbances at 415 nm were recorded after 30 minutes. Each point represents the mean absorbance of sample duplicates with mean of duplicate blanks subtracted for each patient. The IgA antibody levels were very

similar in children (<20 years) as compared to adults, both for males and females (mean for adults 0.79; mean for children .103). The same sera tested for IgA antibodies against BPV were also tested for the presence of IgG antibodies according to Fig. 8, which shows age and sex-related distribution of serum IgG antibodies to PV in a normal population. The same sera tested for IgA antibodies against BPV were tested for the presence of IgG antibodies in a similar way as in Fig. 7 except that an anti-IgG horseradish peroxidase conjugated monoclonal antibody was used. The IgG antibodies were strongly elevated in children as compared to adults ($p<0.0015$). There was also a tendency, however not significant, that IgG anti-BPV titers were elevated in normal males as compared to females ($0.1>p>0.05$).

Onehundred and thirthy-nine sera from healthy adult women, 13 sera from women with CIN grade 1, 16 sera from women with CIN grade 2, 16 sera from women with CIN grade 3 and 69 sera from women with invasive squamous cell carcinoma of the cervix uteri (SCC) were analyzed for IgA, IgG and IgM antibodies to PV. For the statistical analysis the different CIN groups and the SCC group were combined to one single cervical neoplasia group. The IgA antibody levels were significantly increased in the CIN or carcinoma patient group, compared to the age and sex matched controls without known CIN ($p<0.025$) as shown in Fig. 9, wherein detection of IgA antibodies against PV in serum from patients with CIN or carcinoma of the cervix uteri is illustrated. Sera were diluted 1:20 and added to plates coated with BPV. IgA antibodies were detected with a horseradish peroxidase labeled monoclonal antibody against human IgA. After the addition of substrate the absorbances

were read at 415 nm after 60 minutes. Each point represent the mean absorbance of duplicates with mean of duplicate for serum blanks subtracted. Compared to the "No CIN" group, the titers in the CIN and carcinoma group are elevated ($p<0.025$).

The IgG antibody titers against PV were significantly decreased in cervical neoplasia patients compared to controls ($p<0.001$) as illustrated in Fig. 10, illustrating detection of IgG antibodies to PV in serum from patients with CIN or carcinoma of the cervix uteri. Same sera as in Fig. 9 were tested for the presence of IgG antibodies against BPV using a rabbit anti-human IgG-alkaline phosphatase conjugate. After addition of substrate the absorbances were recorded at 405 nm after 90 minutes.

There was also a significant decrease in the IgM titers to PV in the cervical neoplasia patient group compared to the controls ($p<0.005$) according to FIG. 11, which illustrates the detection of IgM antibodies to PV in serum from patients with CIN or carcinoma of the cervix uteri. Same sera as in FIG. 9 were also tested for the presence of IgM antibodies against PV. As conjugate an anti-human IgM glucose oxidase conjugate was used and after development the plates were read at 415 nm after 60 minutes. When the difference between the IgA and IgG response (IgA-IgG) was compared for the group without known CIN and the CIN or carcinoma group, a striking elevation for the CIN or carcinoma patient group was noted ($p<0.0001$). FIG. 12 illustrates the difference between the IgA and IgG response (IgA-IgG) to PV in relation to disease. Same absorbance values as recorded in figures 9 and 10 were plotted as IgA subtracted with IgG.

All HPV genomes have at least eight potentially protein-encoding regions, open reading frames (ORFs). Two ORFs, L1 and L2, have been demonstrated to encode viral capsid proteins. The L1 protein is an approximately 54 kDa abundant capsid protein that is regularly immunogenic for both IgG and IgA antibodies. Antibodies to the L1 protein have virus-neutralizing activity, at least for BPV-1. Several studies using monoclonal antibodies or bacterially expressed fusion proteins have shown that the L1 protein both has epitopes in common for all types of PV, the so-called group-specific epitopes, as well as epitopes specific for each HPV type. One group-specific and one type-specific epitope of L1 in HPV 6 have been mapped using a set of small fusion proteins. The L2 ORF encodes an approximately 70 kDa capsid protein, by us referred to as the approximately 64-kDa protein, that is comparatively low in abundance. The L2 protein has been reported to contain type-specific epitopes. In order to obtain a more complete map of the linear epitopes of the HPV 16 major capsid proteins, the entire amino acid sequences of these two proteins were synthesized as a set of 20 amino acids synthetic peptides with 5 amino acids overlap. The positions of the sequence-specific epitopes that reacted with IgA, IgG or IgM antibodies in the sera from patients with HPV 16-carrying cervical neoplasia are reported in FIG. 13-19.

Sixty-six 20 amino acids peptides with a 5 amino acids overlap to each other were synthesized according to the deduced amino acid sequence of the L1 and L2 ORFs of HPV 16. For denoting the position in the protein, the putative initiation codon was assigned to be amino acid number 1. Synthetic peptide number

1 corresponds to amino acids 2-21 in the L1 ORF, peptide number 2 to amino acids 17-36, number 3 to amino acids 32 to 51 and so on until the L1 carboxy-terminal peptide (number 35) which corresponds to amino acids 512-531. Peptide number 36 corresponds to the amino terminus of the L2 ORF (amino acids 2-21), number 37 is amino acids 17-36 and so on. The two peptides at the L2 carboxyterminus (number 65 and 66) were synthesized as 21 residues peptides, resulting in that their amino acid positions are 437-457 and 453-473, respectively. The amino acid sequences of the most immunoreactive peptides are listed in table 1. The symbols therein are explained by table 2. The amino acid sequences of all peptides used in FIG. 13-19 are shown in table 3. Peptides were synthesized using t-Boc amino acids (Bachem AG, Bubendorf, Switzerland) and p-methylbenzhydrylamine resin (Fluka AG, Buchs, Switzerland) according to the multiple solid phase peptide synthesis method. Removal of the protecting groups from the formyl-tryptophane and methionine sulfoxide residues was achieved by cleavage with 25 % hydrogen fluoride. The peptides were then cleaved from the resin with liquid hydrogen fluoride using a multivessel apparatus.

Sixty-four sera from patients with either carcinoma in situ (CIN grade 3) or invasive cervical carcinoma were obtained. The corresponding cervical biopsies had been analyzed for presence of HPV DNA either by Southern blot (32 cases) or by dot-blot (32 cases) hybridization with 32-P-labelled probes of cloned HPV 16 and HPV 18, as described. HPV 16 had been detected in 32 cases and HPV 18 in 4 cases. Thirty sera from the patients with HPV 16carrying cervical neoplasia were available in sufficient

amounts to be tested with all the different synthetic peptides.

22 sera from patients with other tumors were obtained. 38 sera were obtained from healthy women.

The synthetic peptides were diluted in 10 mM carbonate buffer, pH 9.6 and added to half-area 96-well microtiter plates (Costar) at a concentration of 20 µg peptide/ml. The plates were kept at room temperature over night. After one wash with PBS-0.05 % Tween 20 (PBS-T) the plates were blocked with 10 % lamb serum (heat inactivated; Flow) in PBS and incubated for 60 minutes at 37°C. The blocking solution was then discarded and the plates tapped thoroughly against paper. Human sera were diluted 1:30 in 10 % lamb serum/PBS, added to the plates and allowed to react for 120 minutes at 37°C. The plates were then washed five times with PBS-T. To detect bound antibodies there was a horseradish peroxidase labeled monoclonal antibody against human IgA diluted 1:500 in 10 % lamb serum/PBS incubated on the plates for 120 minutes at 37°C. The plates were then washed five times with PBS-T and developed with 20 mg/ml 2,2'-azino-di (3-ethylbenzthiazolin-sulfonat (6)) deammonium salt (ABTS) diluted 1:50 in 0.1 M citrate buffer, pH 4 with 0.9 % hydrogen peroxide. The absorbances were recorded at 415 nm after 60 minutes. For detection of IgG, the plates were washed and blocked with 10 % lamb serum-PBS for 60 minutes at 37°C. Thereafter, a rabbit anti-human IgG-alkaline phosphatase conjugate (Dako), diluted 1:1000 in 10 % lamb serum-PBS, was applied for 120 minutes at 37°C. After washing five times with PBS-T and once with 0.1 M diethanolamine buffer, pH 9.6, 1 mg/ml phosphatase substrate (Sigma) in 0.1 M diethanolamine buffer, pH 9.6/1mM $MgCl_2$

was added and the plates were read at 405 nm after 90 minutes. For detection of IgM antibodies, the plates were washed and blocked as described above and then incubated with an anti-human IgM-glucose oxidase conjugate (Sera-lab), at dilution 1:800 in 10 % lamb serum-PBS, for 120 minutes. As substrate there was used 0.36 mg/ml of ABTS, 2.4 % glucose, 8 µg/ml horseradish peroxidase (Sigma) in 0.1 M phosphate buffer, pH 6.0. The plates were read at 415 nm after 60 minutes. An absorbance of 0.1 or more above background (same serum on uncoated wells) was considered a positive reaction. As an internal standard, the 30 HPV 16-positive sera were in each test reacted with the known antigenic peptide HKSAIVTLTYDSEWQRDQC from the E2 ORF of HPV 16. The ELISA absorbances were adjusted relative to the internal standard to compensate for possible inter-assay variation.

The ELISA absorbances, with absorbances of the same serum reacted with uncoated wells subtracted, were analysed for statistically significant differences between the HPV 16-carrying cervical neoplasia patient group and the control group by a two-sided, non-parametric ranking test (Mann-Whitney test).

The complete amino acid sequence of the L1 and L2 proteins of HPV type 16 were synthesized as a set of 66 synthetic peptides, 20 amino acids long and with a 5 residues overlap to each other. All peptides were tested in ELISA for reactivity with either IgA, IgG or IgM antibodies in 30 sera from HPV 16-carrying cervical neoplasia patients. Several regions of the L1 protein were regularly reactive with IgA antibodies present in these sera as illustrated in FIG. 13, which shows IgA reactivity to

the L1 protein. Each point represents the absorbance value (OD) for one serum reacted with the peptide whose number is given below the abscissa. The absorbance value for the same serum when reacted with uncoated wells has been subtracted. Thirty sera from patients with HPV 16-carrying cervical neoplasia were diluted at 1:30 and reacted with 35 twenty residues synthetic peptides representing the entire amino acid sequence of the L1 ORF of HPV 16. Bound IgA antibodies were detected with an IgA (alpha-chain)-specific enzyme conjugated monoclonal antibody. The IgA response was especially strong against the peptides deduced from an internal region of the protein spanning amino acids 167 up to 271, corresponding to peptides 12-18. Outside this major immunoreactive region there were several additional immunoreactive epitopes, notably the amino terminus of the protein (peptide 1), peptide 8 (amino acids 102-121) and several epitopes positioned on the carboxyterminal side of the major immunoreactive region, notably peptides 21 and 31 (corresponding to amino acids 302-321 and to 452-471, respectively).

In contract, very few peptides deduced from the L2 protein were reactive with IgA antibodies in these sera as shown in FIG. 14, which shows IgA reactivity to the L2 protein. Similar experiment as in FIG. 13, except that the sera were reacted with 31 twenty residues synthetic peptides representing the entire amino acid sequence of the L2 ORF of HPV 16. The major epitope was positioned in the middle of the protein (peptide 49, amino acids 197-216). Other epitopes were also detected in the amino-terminal part of the protein (peptides 37-38, amino acids 17-41), whereas the carboxyterminal part of L2 was less reactive.

The IgG reactivity of the L1 protein was less than the IgA reactivity. Reference is made to FIG. 15, which shows IgG reactivity to the L1 protein. Similar experiment as in FIG. 13, except that an IgG (gamma-chain)-specific enzyme conjugated rabbit antibody was applied. Although the major IgA-immunoreactive region (peptides 12-18) was also immunoreactive with IgG antibodies, the IgG reactivity was equally strong also with several epitopes outside this region, e.g. with peptide 24 (amino acids 347-366).

The L2 protein had a few IgG-reactive epitopes as illustrated in FIG. 16, which shows IgG reactivity to the L2 protein. Similar experiment as in FIG. 13, except that an IgG (gamma-chain)-specific enzyme conjugated rabbit antibody was applied and that the sera were reacted with 31 twenty residues synthetic peptides representing the entire amino acid sequence of the L2 ORF of HPV 16. This resembles what we found for the IgA reactivity of this protein. Peptide 49, which was the major IgA reactive epitope of this protein, was also found to be the major IgG-reactive epitope. The IgA-reactive epitope at the aminoterminus of L2 (peptides 37-38) was also found to be IgG-reactive (FIG. 4).

The IgM reactivity to the L1 protein was scattered among several epitopes along the entire length of the protein as illustrated in FIG. 17, which shows IgM reactivity to the L1 protein. Similar experiment as in FIG. 13, except that an IgM (mu-chain)-specific enzyme conjugated goat antibody was applied. Interestingly, the main IgM reactivity was found against peptides that were not very immunoreactive with IgG or IgA antibodies, e.g. peptides 9, 13 and 23 (amino acid positions 122-142, 182-202 and 332-352).

The IgM immunoreactivity of the L2 protein resembled that for the IgA and IgG in that the L2 peptides were much less reactive than the L1 peptides. Reference is made to FIG. 18, which shows IgM reactivity to the L2 protein. Similar experiment as in FIG. 13, except that an IgM (mu-chain)-specific enzyme conjugated goat antibody was applied and that the sera were reacted with 31 twenty residues synthetic peptides representing the entire amino acid sequence of the L2 ORF of HPV 16. Actually, not a single L2 peptide was immunoreactive with IgM in more than 4 out of 30 sera. The seven most immunoreactive peptides (peptides 8, 12, 13, 14, 16, 17, and 24) were also tested for IgA, IgG and IgM reactivity with a panel of 60 control sera, of which 22 were obtained from patients with irrelevant tumors and 38 were obtained from healthy donors. Most of these peptides showed significant immunoreactivity only with less than 10 % of these control sera as illustrated in FIG. 19, which shows disease-associated reactivity of synthetic peptides. Four of the highly immunoreactive synthetic peptides that had striking differences in reactivity between 30 HPV-16-carrying cervical neoplasia sera (HPV 16 SCC) and a control group of 60 sera from patients with other tumors or from healthy donors (Control) are shown in FIG. 19. Each point denotes the absorbance value in ELISA with the absorbance value on uncoated wells subtracted. A high sensitivity for detection of HPV 16-carrying cervical neoplasia was seen with the IgM reactivity to peptide 8: 70 %. The specificity of this test was 95 % (3/60 control sera were reactive). The IgG reactivity to peptide 24, showed a high specificity: 97 % (2/60 control sera were reactive), but a lower sensitivity, 53 % (16/30

patient sera reacted). The IgA reactivities to peptides 14 and 16 both had sensitivities around 60 % and specificities just above 90 % (FIG. 19), and the immunoreactivity among the positive sera was among the highest detected (compare FIGS. 1-6).

By showing that a peptide is immunoreactive, the inventors have defined that it contains an epitope reactive with human sera. The epitope contained within this peptide sequence is not absolutely dependent on the exact sequence of the peptide, but can also be contained in a variety of minor modifications of the original peptide. Such modifications include extensions, truncations, cyclizations and amino acid substitutions. Sometimes the question arises if such a modified peptide should be considered a new peptide containing a new epitope. By competitive immunoassays with the original peptide and the modification thereof, it is straightforward to determine if the modified peptide is substantially immunoreactive with antibodies to the original peptide and thus contains the same epitope. It should be emphasized that a peptide can be produced in many different ways. Herein peptide synthesis by organic chemistry methods has been used, but the same peptides can also be produced by many other means for example by recombinant DNA expression systems.

It is understood that the herein contained description of the methods is intended to exemplify, but not limit, the present invention. An immunoassay can for example be performed in a variety of different ways. Detection of the antibodies that have bound to the specific antigen can for example be achieved with various antibodies to antibodies (anti-antibodies) or other compounds with affinity

for antibodies, such as protein A or protein G. These reagents can be labelled in many different ways, for example radioactively (radioimmunoassay), with fluorescein (fluoroimmunoassay) or enzymatically (enzyme-linked immunoassay, ELISA or EIA). A special case of enzymatic immunoassay is when the antigen-antibody complexes are detected on tissue sections. Such a procedure is instead referred to as immunostaining or immunohistocytochemistry, although the underlying principle is the similar as for ELISA.

An ELISA procedure can also be carried out in a variety of formats. Methods for enhancement of ELISA sensitivity using several layers of anti-antibodies, avidin-biotin complexes and enzyme-anti-enzyme antibody complexes are well known in the art. The solid support for fixation of antigen is usually plastic, as described here, but a variety of other solid supports such as latex or agarose have been described. It is also not necessary for the antigen to be directly fixed onto the solid support. There is for example a commonly used ELISA format that fixes the specific antigen to the solid support via a solid-phase-fixed antibody to the antigen, so-called catching antibody ELISA or sandwich ELISA.

A special case of immunoassay which involves a blotting (transfer) of antigen to a solid support in sheet format is termed immunoblotting. Typically, the solid support is nitrocellulose or nylon sheets, but other supports have been described. It is also a typical feature of this method that, prior to blotting, the antigens are separated according to size by gel electrophoresis or similar methods. Detection of antibodies bound to the specific antigen on the sheet can be carried out in similar ways as for other immunoassays. The here described

detection using an anti-antibody, a biotin-avidin complex enhancement step and an enzymatic labelling is just one example of such a detection.

For diagnostic methods in general it is well known that a combination of several diagnostic methods produces a diagnostic method with better sensitivity and/or specificity than the individual tests contained in the combination. It is self-evident that any of the here described antibody tests could be combined with each other, or with other tests, to produce a combined diagnostic test with optimal sensitivity and specificity.

Table 1A

	E490	
IgG	serum	secretions
19084	.368	.184
19085	.592	.173
19086	1.041	.306
19087	.230	.073
19088	.770	.188
19089	.240	.180
19090	.253	.294
19091	.210	.032
19092	.198	.060
19093	.660	.195
19094	.945	.281
19095	.224	.228
19096	.280	.278
19097	.259	.226
19098	.222	.134
IgA	serum	secretions
19084	.298	.090
19085	.199	.072
19086	.327	.131
19087	.137	.056
19088	.235	.118
19089	.140	.053
19090	.153	.070
19091	.122	.076
19092	.119	.062
19093	1.021	.394
19094	.140	.066
19095	.073	.099
19096	.136	.098
19097	.128	.096
19098	.098	.050

Measurement of IgA and IgG antibodies to PV in serum and secretions. Extinction coefficients from ELISA results based on 15 patients. Part of this information is depicted in figures 2, 3 and 4. Statistical analysis of the data show that measurement of IgA antibodies to PV, either in serum or secretions, is a test that is not correlated to the previously described test for IgG antibodies to PV, either in serum or secretions.

Table 1

Peptide No.	Peptide sequence	IgA	IgG	IgM
8	NKFGFPDTSFYNPDTQRLVW	<0.05	<0.0001	<0.0001
12	VDNRECISMDYKQTQLCLIG	<0.002	<0.0001	<0.01
13	LCLIGCKPPIGEHWGKGSPC	<0.02	<0.0001	NS
14	KGSPCTNVAVNPGDCPPLEL	<0.0005	<0.0001	<0.0001
16	VHTGFGAMDFTTLQANKSEV	<0.0001	<0.0001	<0.0001
17	NKSEVPLDICTSICKYPDYI	<0.01	NS	<0.0001
24	NGICWGNQLFVTVVDTTRST	<0.002	<0.0001	<0.001

Detection of significantly elevated antibody titers against HPV 16 synthetic peptides among sera from 30 patients with HPV 16-carrying cervical neoplasia as compared to a control group of 60 sera from patients with other tumors or from healthy donors. Figures denote the p-values for significant differences among these two parameters (Mann-Whitney test).

NS = Not significant

Table 2

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	L-tyrosine
G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Try	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
C	Cys	L-cysteine

Table 3

Amino acid sequences of the synthetic peptides used in the experiments depicted in figures 13 - 19.

Pep No.	Sequence	Pep No.	Sequence
1	QVTFIYILVITCYENDVNVY	41	TRPPTATDTLAPVRPPLTVD
2	DVNVYHIFFQMSLWLPSEAT	42	PLTVDPVGPSDPSIVSLVEE
3	PSEATVYLPPVPVSKVVSTD	43	SLVEETSFIDAGAPTSVPSI
4	VVSTDEYVARTNIYYHAGTS	44	SVPSIPPDVSGFSITTSTDT
5	HAGTSRLLAVGHPYFPIKKP	45	TSTDTTPAILDINNTVTTVT
6	PIKKPNNNKILVPKVSGLQY	46	VTTVTTHNNPTFTDPSVLQP
7	SGLQYRVFRIHLPDPNKFGF	47	SVLQPPTPAETGGHFTLSSS
8	NKFGFPDTSFYNPDTQRLVW	48	TLSSSTISTHNYEEIPMDTF
9	QRLVWACVGVEVGRGQPLGV	49	PMDTFIVSTNPNTVTSSTPI
10	QPLGVGISGHPLLNKLDDTE	50	SSTPIPGSRPVARLGLYSRT
11	LDDTENASAYAANAGVDNRE	51	LYSRTTQQVKVVDPAFVTTP
12	VDNRECISMDYKQTQLCLIG	52	FVTTPTKLITYDNPAYEGID
13	LCLIGCKPPIGEHWGKGSPC	53	YEGIDVDNTLYFSSNDNSIN
14	KGSPCTNVAVNPGDCPPLEL	54	DNSINIAPDPDFLDIVALHR
15	PPLELINTVIQDGDMVHTGF	55	VALHRPALTSRRTGIRYSRI
16	VHTGFGAMDFTTLQANKSEV	56	RYSRIGNKQTLRTRSGKSIG
17	NKSEVPLDICTSICKYPDYI	57	GKSIGAKVHYYYDLSTIDPA
18	YPDYIKMVSEPYGDSLFFYL	58	TIDPAEEIELQTITPSTYTT
19	LFFYLRREQMFVRHLFNRAG	59	STYTTTSHAASPTSINNGLY
20	FNRAGTVGENVPDDLYIKGS	60	NNGLYDIYADDFITDTSTTP
21	YIKGSGSTANLASSNYFPTP	61	TSTTPVPSVPSTSLSGYIPA
22	YFPTPSGSMVTSDAQIFNKP	62	GYIPANTTIPFGGAYNIPLV
23	IFNKPYWLQRAQGHNNGICW	63	NIPLVSGPDIPINITDQAPS
24	NGICWGNQLFVTVVDTTRST	64	DQAPSLIPIVPGSPQYTIIA
25	TTRSTNMSLCAAISTSETTY	65	YTIIADAGDFYLHPSYYMLRK
26	SETTYKNTNFKEYLRHGEEY	66	YMLRKRRKRLPYFFSDVSLAA
27	HGEEYDLQFIFQLCKITLTA		
28	ITLTADVMTYIHSMNSTILE		
29	STILEDWNFGLQPPPGGTLE		
30	GGTLEDTYRFVTQAIACQKH		
31	ACQKHTPPAPKEDDPLKKYT		
32	LKKYTFWEVNLKEKFSADLD		
33	SADLDQFPLGRKFLLQAGLK		
34	QAGLKAKPKFTLGKRKATPT		
35	KATPTTSSTSTTAKRKKRKL		
36	RHKRSAKRTKRASATQLYKT		
37	QLYKTCKQAGTCPPDIIPKV		
38	IIPKVEGKTIAEQILQYGSM		
39	QYGSMGVFFGGLGIGTGSGT		
40	TGSGTGGRTGYIPLGTRPPT		

CLAIMS

1. Method for detection of infection with papillomavirus (PV) for diagnostic purposes, specifically for diagnosing PV-associated neoplasia, on a body fluid or tissue, c h a r a c t e r i z e d in that the detection is effected by ascertaining the presence in the body fluid or tissue of IgA antibodies to the L2-encoded approximately 64 kDa papillomavirus protein, the L1-encoded approximately 54 kDa papillomavirus protein, as well as any type of antibodies to an approximately 28 kDa papillomavirus virion protein, to an approximately 14 kDa papillomavirus virion protein, or to a peptide having an amino acid sequence represented by a formula selected from the group consisting of

QVTFIYILVITCYENDVNVY
PSEATVYLPPVPVSKVVSTD
VVSTDEYVARTNIYYHAGTS
HAGTSRLLAVGHPYFPIKKP
PIKKPNNNKILVPKVSGLQY
SGLQYRVFRIHLPDPNKFGF
NKFGFPDTSFYNPDTQRLVW
QRLVWACVGVEVGRGQPLGV
VDNRECISMDYKQTQLCLIG
LCLIGCKPPIGEHWGKGSPC
KGSPCTNVAVNPGDCPPLEL
PPLELINTVIQDGDMVHTGF
VHTGFGAMDFTTLQANKSEV
NKSEVPLDICTSICKYPDYI
YPDYIKMVSEPYGDSLFFYL
YIKGSGSTANLASSNYFPTP
YFPTPSGSMVTSDAQIFNKP
IFNHPYWLQRAQGHNNGICW
NGICWGNQLFVTVVDTTRST
TTRSTNMSLCAAISTSETTY

SETTYKNTNFKEYLRHGEEY
ITLTADVMTYIHSMNSTILE
GGTLEDTYRFVTQAIACQKH
ACQKHTPPAPKEDDPLKKYT
LKKYTFWEVNLKEKFSADLD
SADLDQFPLGRKFLLQAGLK
QAGLKAKPKFTLGKRKATPT
QLYKTCKQAGTCPPDIIPKV
IIPKVEGKTIAEQILQYGSM
PMDTFIVSTNPNTVTSSTPI
GKSIGAKVHYYYDLSTIDPA
NNGLYDIYADDFITDTSTTP

or to such modifications of any of the peptides of said group which are substantially immunoreactive with antibodies to the original peptide.

2. Method as in claim 1, wherein said antibodies are of human origin and the infection to be detected is HPV infection or HPV-associated disease.

3. Method as in claim 1, wherein the presence of antibodies is ascertained by immunoassay.

4. Method as in claim 3, wherein the immunoassay is ELISA.

5. Method as in claim 3, wherein the immunoassay is immunoblotting.

6. Method as in claim 1, wherein the detection is effected on a secretion.

7. Method as in claim 6, wherein the detection is effected on cervical secretion.

8. Method as in claim 7, wherein the detection is made for diagnosis of cervical neoplasia.

9. Method as in claim 6, wherein the secretion is taken on a swab, brush, spatula or the like, and is washed off therefrom in a liquid diluent.

10. Method as in claim 1, wherein the detection is effected on serum.

11. Method as in claim 1, where the detection is effected by ascertaining the presence in the body fluid or tissue of IgG antibodies to an approximately 28 kDa papillomavirus virion protein, an approximately 14 kDa virion papillomavirus protein, or to a peptide having an amino acid sequence represented by a formula selected from the group consisting of

PSEATVYLPPVPVSKVVSTD
VDNRECISMDYKQTQLCLIG
KGSPCTNVAVNPGDCPPLEL
YPDYIKMVSEPYGDSLFFYL
YIKGSGSTANLASSNYFPTP
NGICWGNQLFVTVVDTTRST
TTRSTNMSLCAAISTSETTY
SADLDQFPLGRKFLLQAGLK
QLYKTCKQAGTCPPDIIPKV
IIPKVEGKTIAEQILQYGSM
PMDTFIVSTNPNTVTSSTPI
NNGLYDIYADDFITDTSTTP

or to such modifications of any of the peptides of said group which are substantially immunoreactive with antibodies to the original peptide.

12. Method as in claim 11, wherein said IgG antibodies are of human origin and the infection to be detected is HPV infection or HPV-associated disease.

13. Method as in claim 11, wherein the presence of antibodies is ascertained by immunoassay.

14. Method as in claim 11, wherein the immunoassay is ELISA.

15. Method as in claim 11, wherein the immunoassay is immunoblotting.

16. Method as in claim 11, wherein the detection is effected on a secretion.

17. Method as in claim 16, wherein the detection is effected on cervical secretion.

18. Method as in claim 17, wherein the detection is made for diagnosis of cervical neoplasia.

19. Method as in claim 16, wherein the secretion is taken on a swab, brush, spatula or the like, and is washed off therefrom in a liquid diluent.

20. Method as in claim 11, wherein the detection is effected on serum.

21. Method as in claim 1, where the detection is effected by ascertaining the presence in the body fluid or tissue of IgM antibodies to an amino acid sequence represented by a formula selected from the group consisting of

HAGTSRLLAVGHPYFPIKKP
PIKKPNNNKILVPKVSGLQY
SGLQYRVFRIHLPDPNKFGF
NKFGFPDTSFYNPDTQRLVW
QRLVWACVGVEVGRGQPLGV
VDNRECISMDYKQTQLCLIG
LCLIGCKPPIGEHWGKGSPC
KGSPCTNVAVNPGDCPPLEL
NKSEVPLDICTSICKYPDYI
YPDYIKMVSEPYGDSLFFYL
YIKGSGSTANLASSNYFPTP
IFNHPYWLQRAQGHNNGICW
TTRSTNMSLCAAISTSETTY
SETTYKNTNFKEYLRHGEEY
ITLTADVMTYIHSMNSTILE
GGTLEDTYRFVTQAIACQKH
ACQKHTPPAPKEDDPLKKYT
LKKYTFWEVNLKEKFSADLD

or to such modifications of any of the peptides of said group which are substantially immunoreactive with antibodies to the original peptide.

22. Method as in claim 21, wherein said IgM antibodies are of human origin and the infection to be detected is HPV infection or HPV-associated disease.

23. Method as in claim 21, wherein the presence of antibodies is ascertained by immunoassay.

24. Method as in claim 23, wherein the immunoassay is ELISA.

25. Method as in claim 23, wherein the immunoassay is immunoblotting.

26. Method as in claim 21, wherein the detection is effected on a secretion.

27. Method as in claim 26, wherein the detection is effected on cervical secretion.

28. Method as in claim 27, wherein the detection is made for diagnosis of cervical neoplasia.

29. Method as in claim 26, wherein the secretion is taken on a swab, brush, spatula or the like, and is washed off therefrom in a liquid diluent.

30. Method as in claim 21, wherein the detection is effected on serum.

31. Method as in claim 1, where the detection is effected by ascertaining the presence in the body fluid or tissue of IgA antibodies to an approximately 28 kDa papillomavirus virion protein, an approximately 14 kDa papillomavirus virion protein, or to a peptide having an amino acid sequence represented by a formula selected from the group consisting of:

QVTFIYILVITCYENDVNVY
PSEATVYLPPVPVSKVVSTD
VVSTDEYVARTNIYYHAGTS
HAGTSRLLAVGHPYFPIKKP
SGLQYRVFRIHLPDPNKFGF
NKFGFPDTSFYNPDTQRLVW

QRLVWACVGVEVGRGQPLGV
VDNRECISMDYKQTQLCLIG
LCLIGCKPPIGEHWGKGSPC
KGSPCTNVAVNPGDCPPLEL
PPLELINTVIQDGDMVHTGF
VHTGFGAMDFTTLQANKSEV
NKSEVPLDICTSICKYPDYI
YPDYIKMVSEPYGDSLFFYL
YIKGSGSTANLASSNYFPTP
YFPTPSGSMVTSDAQIFNKP
NGICWGNQLFVTVVDTTRST
SETTYKNTNFKEYLRHGEEY
ACQKHTPPAPKEDDPLKKYT
LKKYTFWEVNLKEKFSADLD
QAGLKAKPKFTLGKRKATPT
QLYKTCKQAGTCPPDIIPKV
IIPKVEGKTIAEQILQYGSM
PMDTFIVSTNPNTVTSSTPI
GKSIGAKVHYYYDLSTIDPA

or to such modifications of any of the peptides of said group which are substantially immunoreactive with antibodies to the original peptide.

32. Method as in claim 31, wherein said IgA antibodies are of human origin and the infection to be detected is HPV infection or HPV-associated disease.

33. Method as in claim 31, wherein the presence of antibodies is ascertained by immunoassay.

34. Method as in claim 33, wherein the immunoassay is ELISA.

35. Method as in claim 33, wherein the immunoassay is immunoblotting.

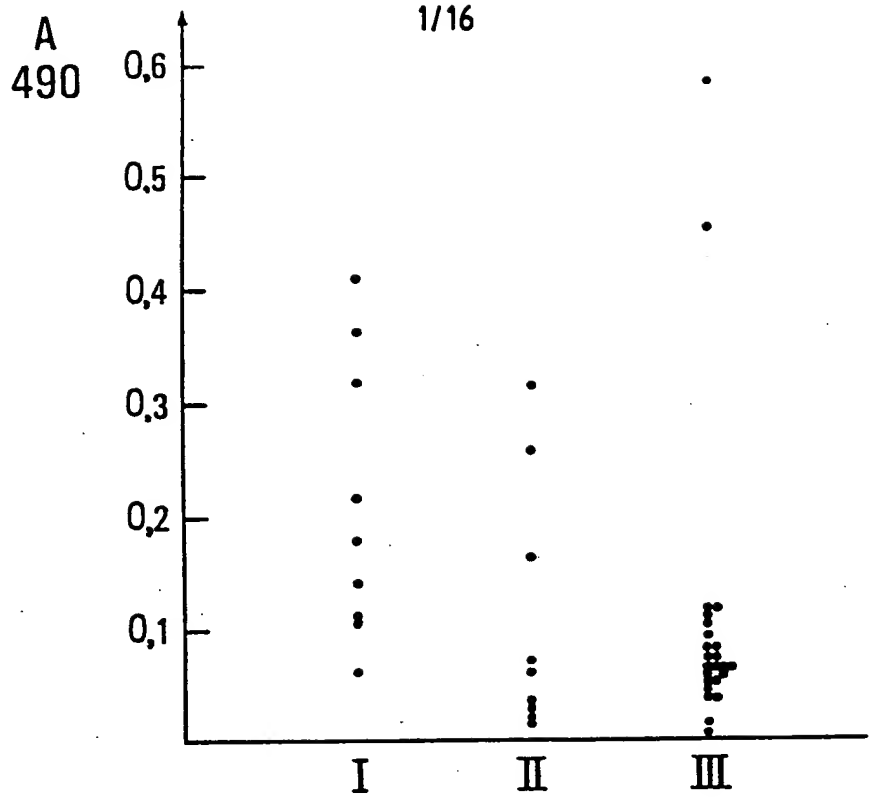
36. Method as in claim 31, wherein the detection is effected on a secretion.

37. Method as in claim 36, wherein the detection is effected on cervical secretion.

38. Method as in claim 37, wherein the detection is made for diagnosis of cervical neoplasia.

39. Method as in claim 36, wherein the secretion is taken on a swab, brush, spatula or the like, and is washed off therefrom in a liquid diluent.

40. Method as in claim 31, wherein the detection is effected on serum.


A
490
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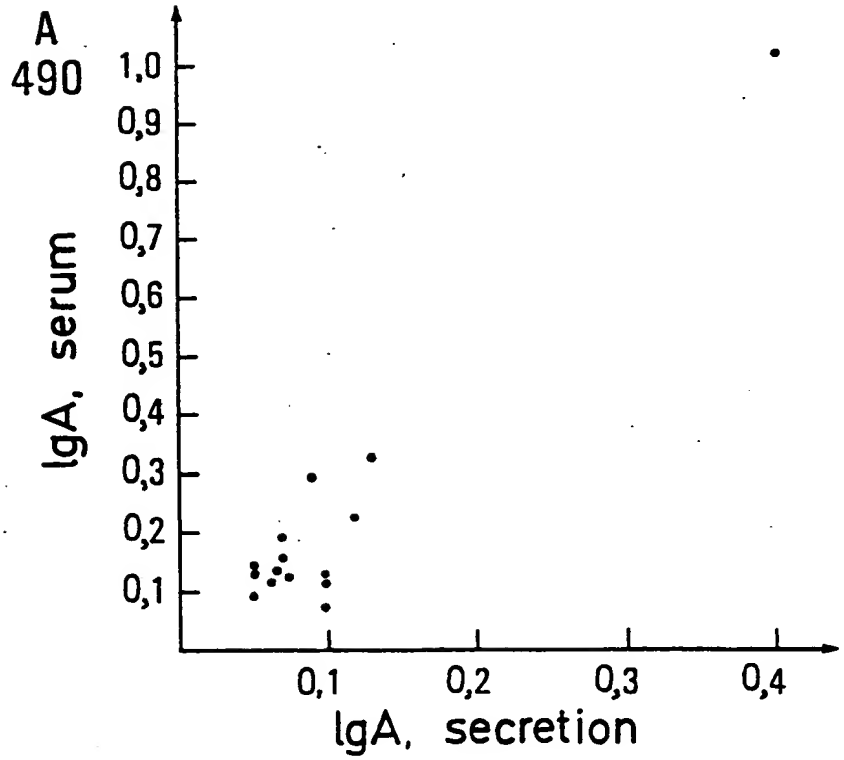
0,5
0,4
0,3
0,2
0,1
I
II
III

FIG. 1


A
490
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
IgA, serum
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0,2
0,3
0,4
IgA, secretion

FIG. 2

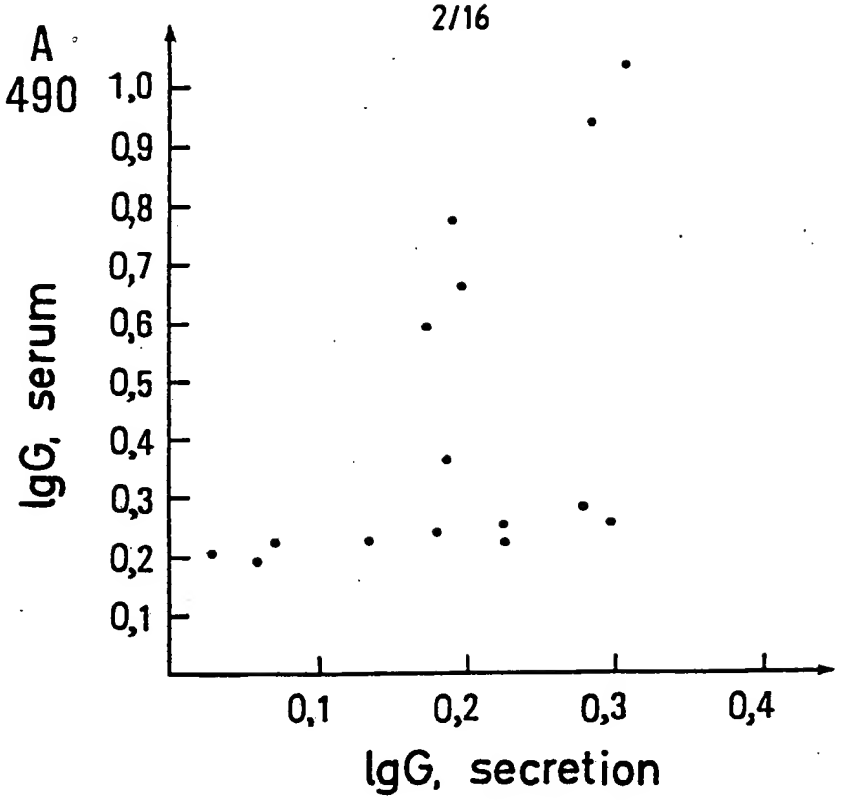

A 490
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
IgG, serum
0,1
0,2
0,3
0,4
IgG, secretion

FIG. 3

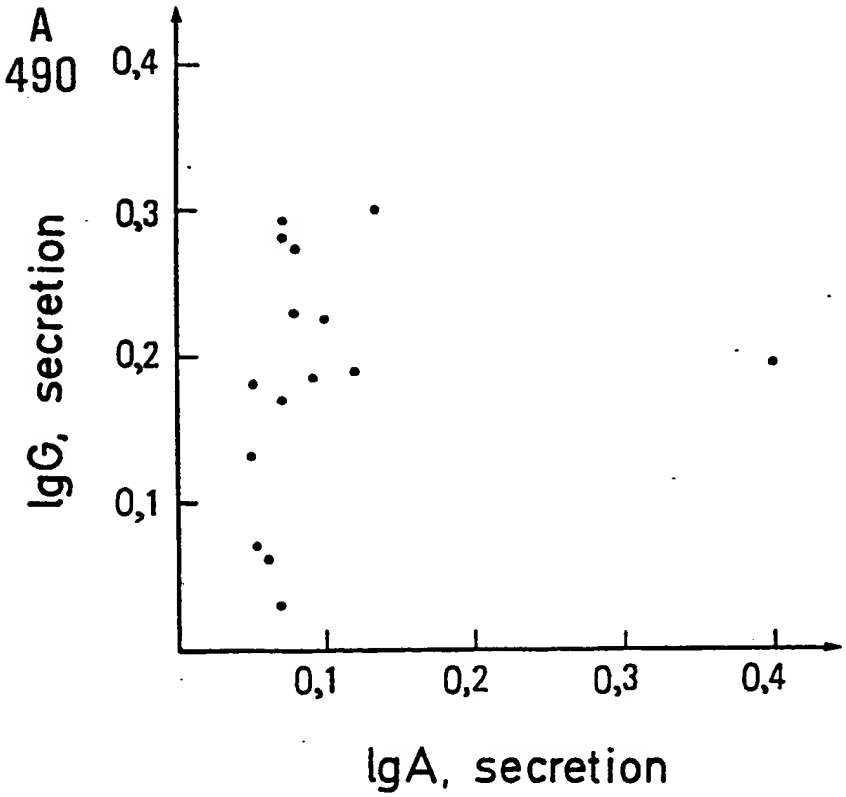

A 490
0,4
0,3
0,2
0,1
IgG, secretion
0,1
0,2
0,3
0,4
IgA, secretion

FIG. 4

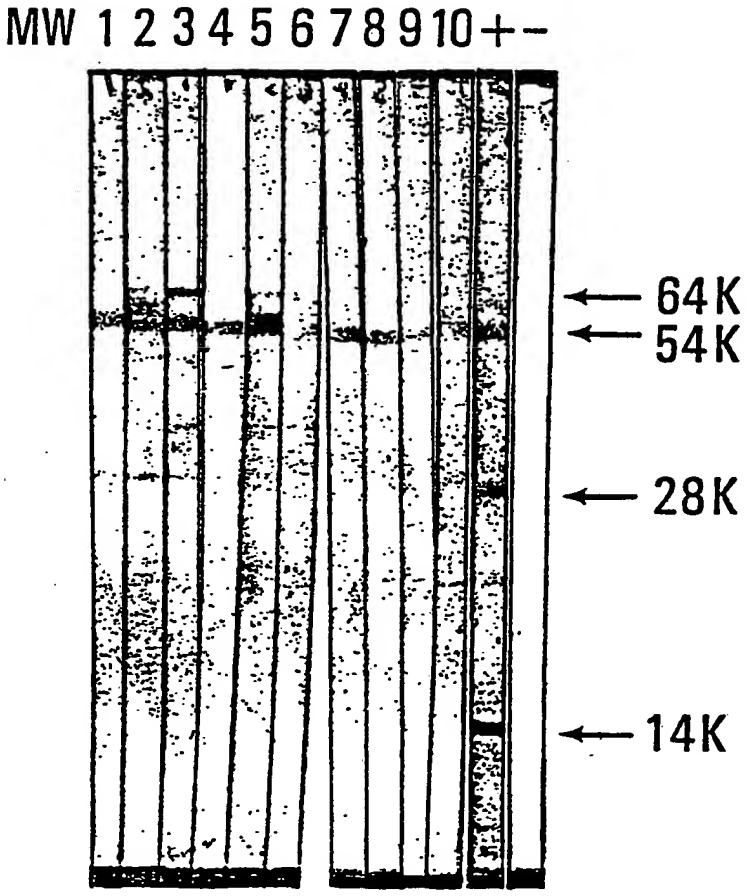


FIG. 5

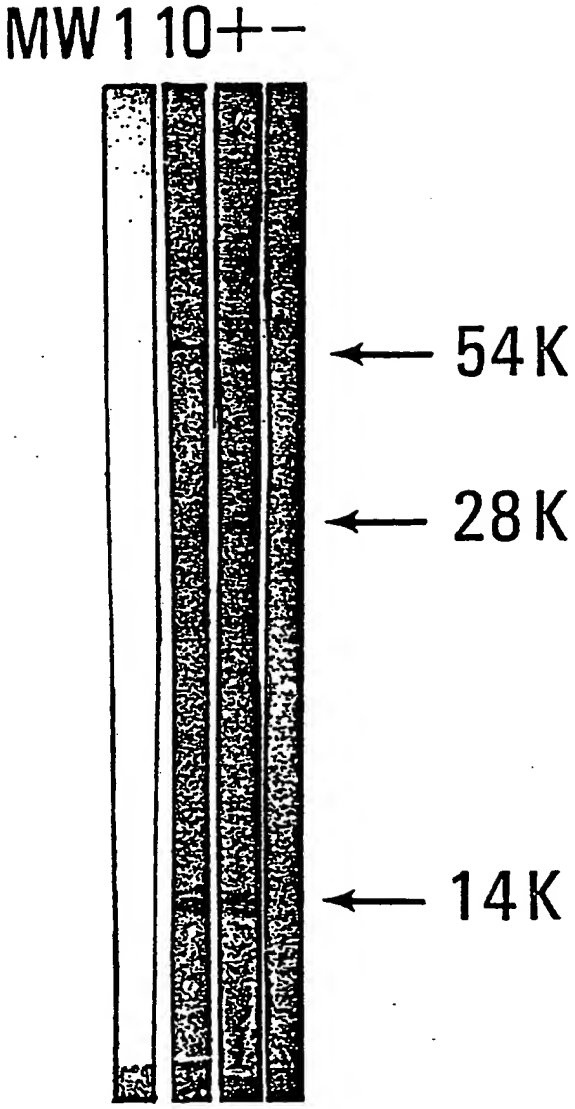


FIG. 6

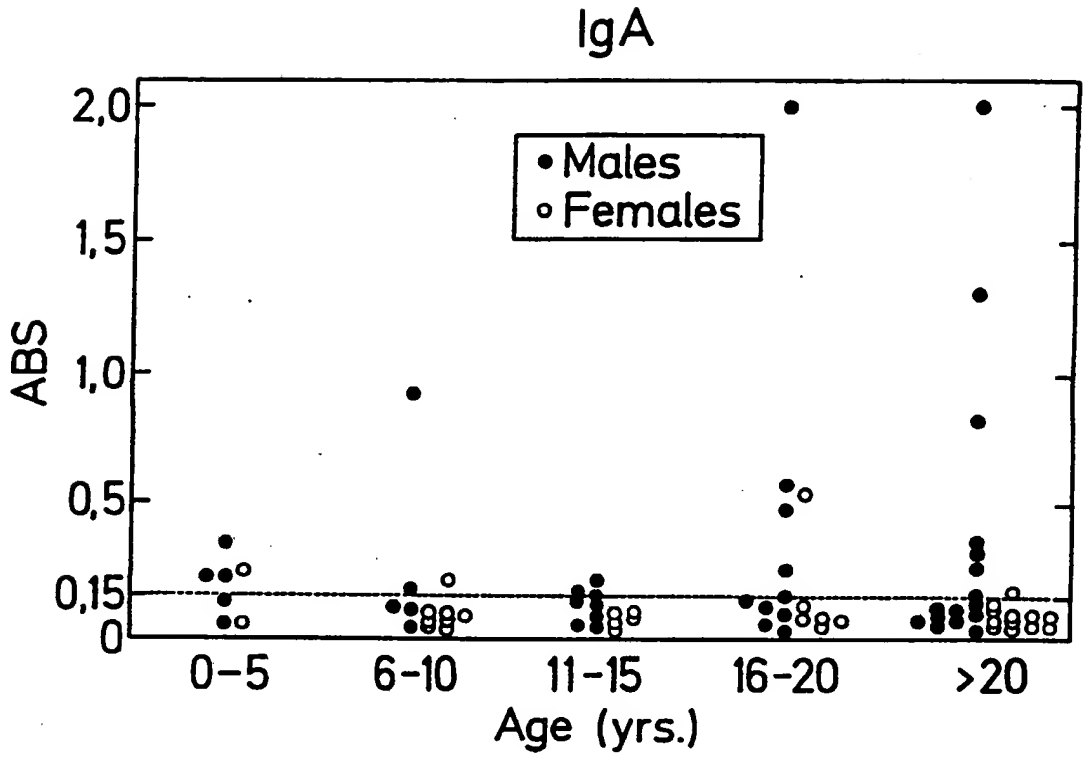

IgA
2,0
1,5
1,0
0,5
0,15
0
ABS
• Males
○ Females
0-5
6-10
11-15
16-20
>20
Age (yrs.)

FIG. 7

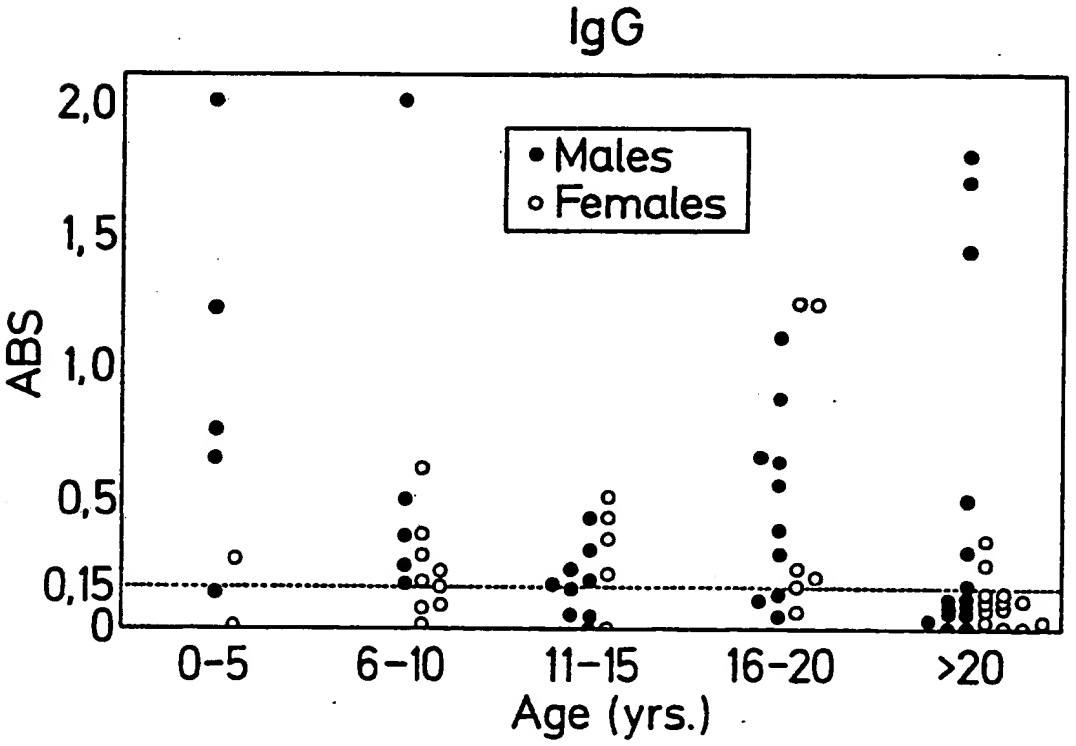

IgG
2,0
1,5
1,0
0,5
0,15
0
ABS
• Males
○ Females
0-5
6-10
11-15
16-20
>20
Age (yrs.)

FIG. 8

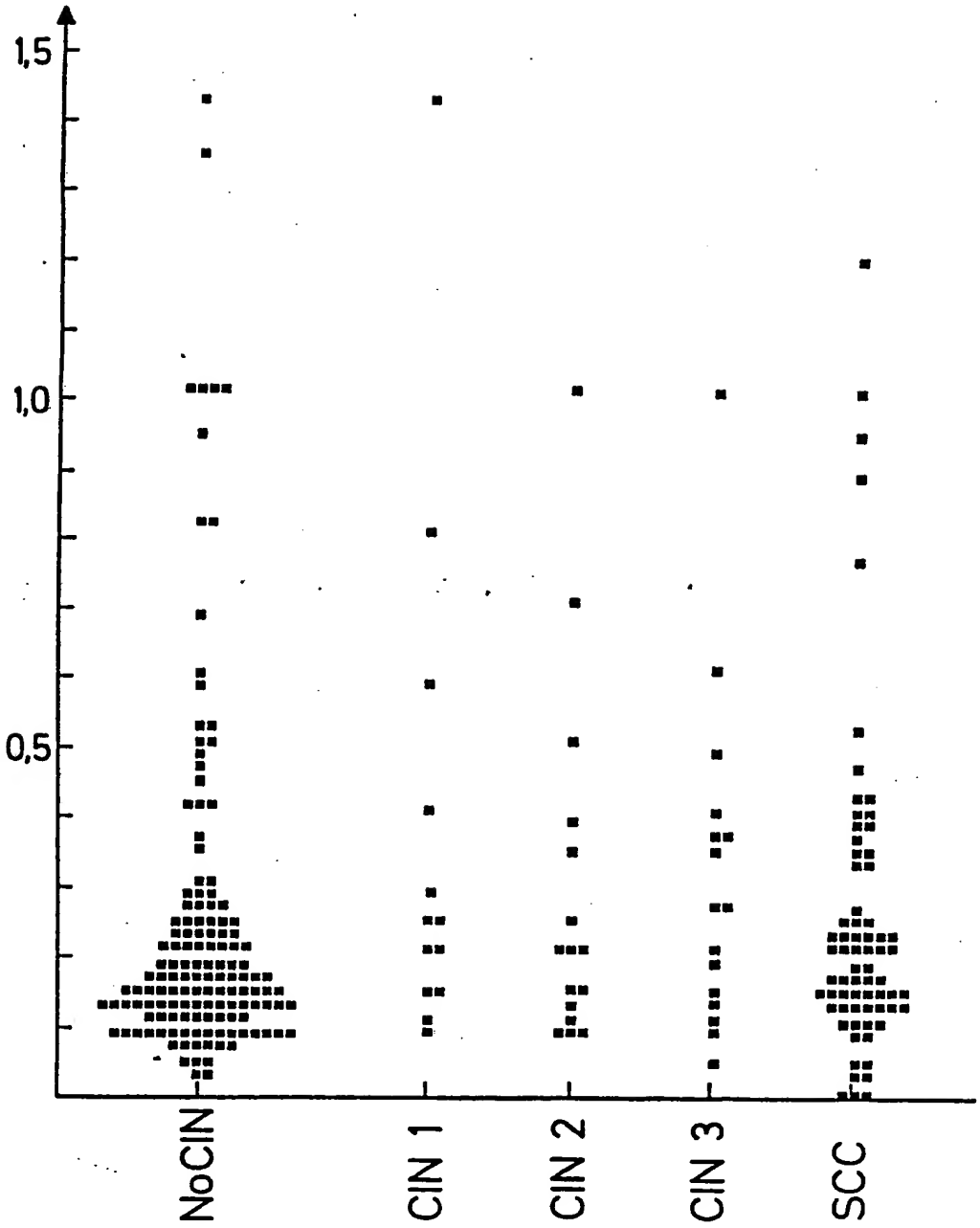


FIG. 9

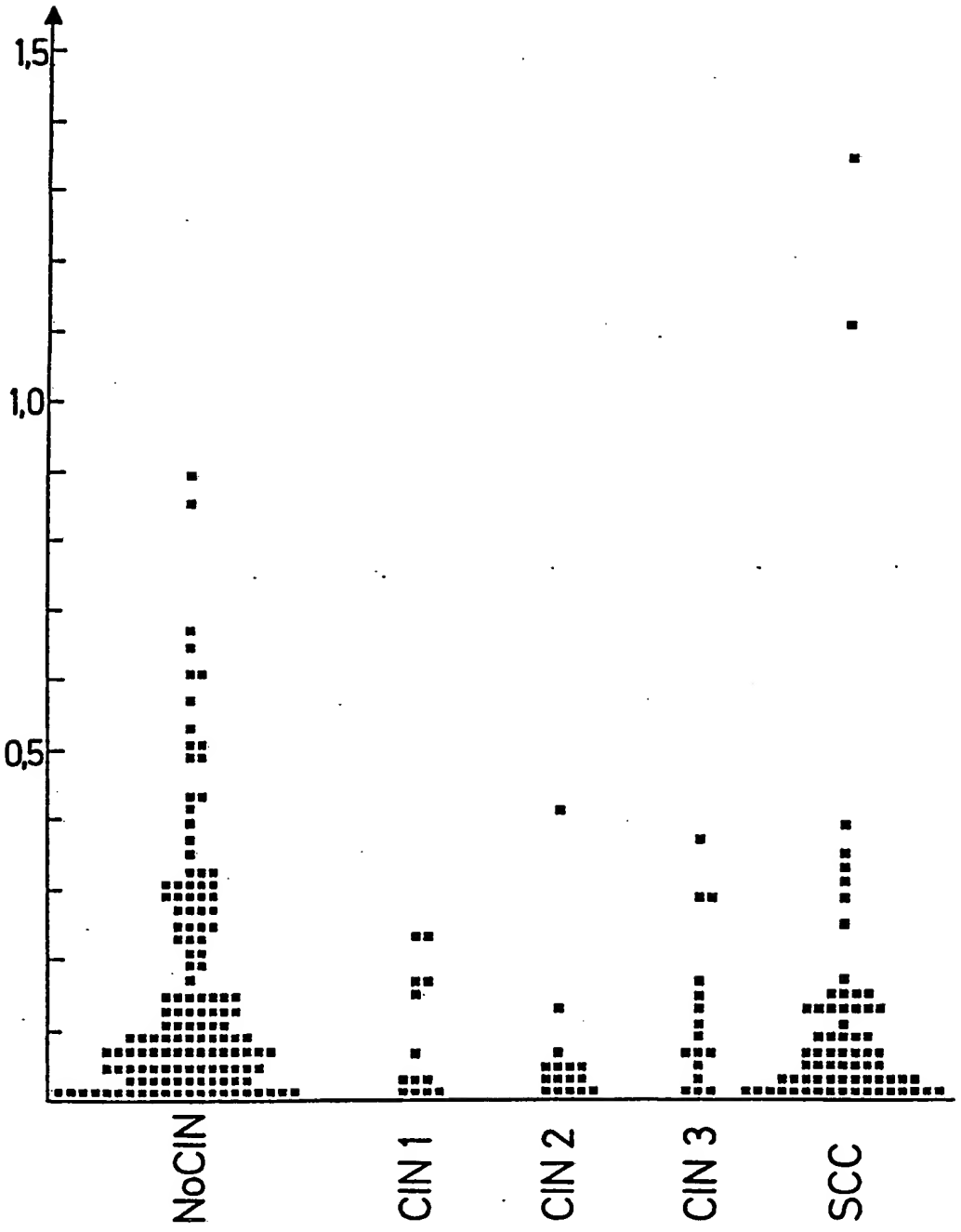


FIG. 10

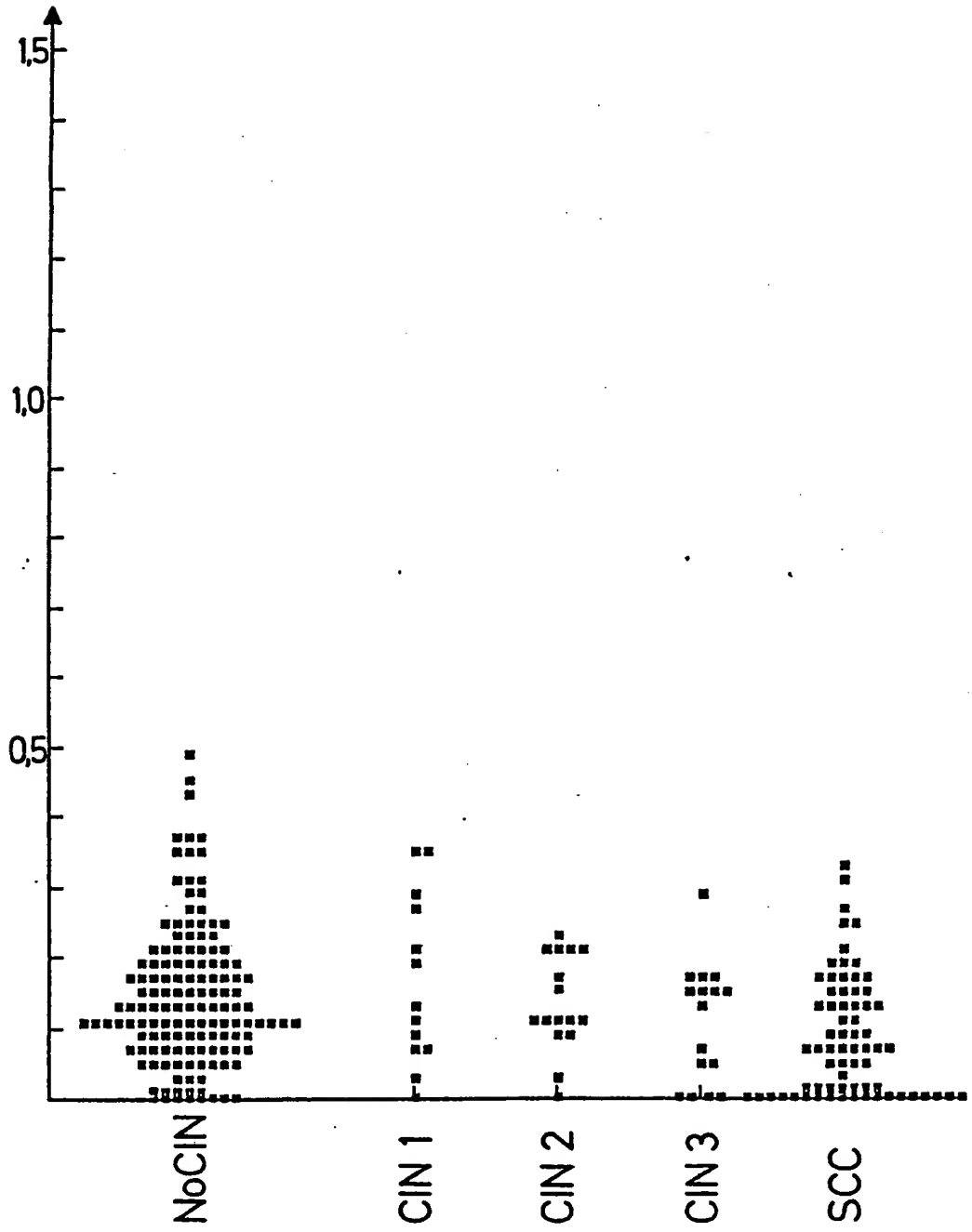
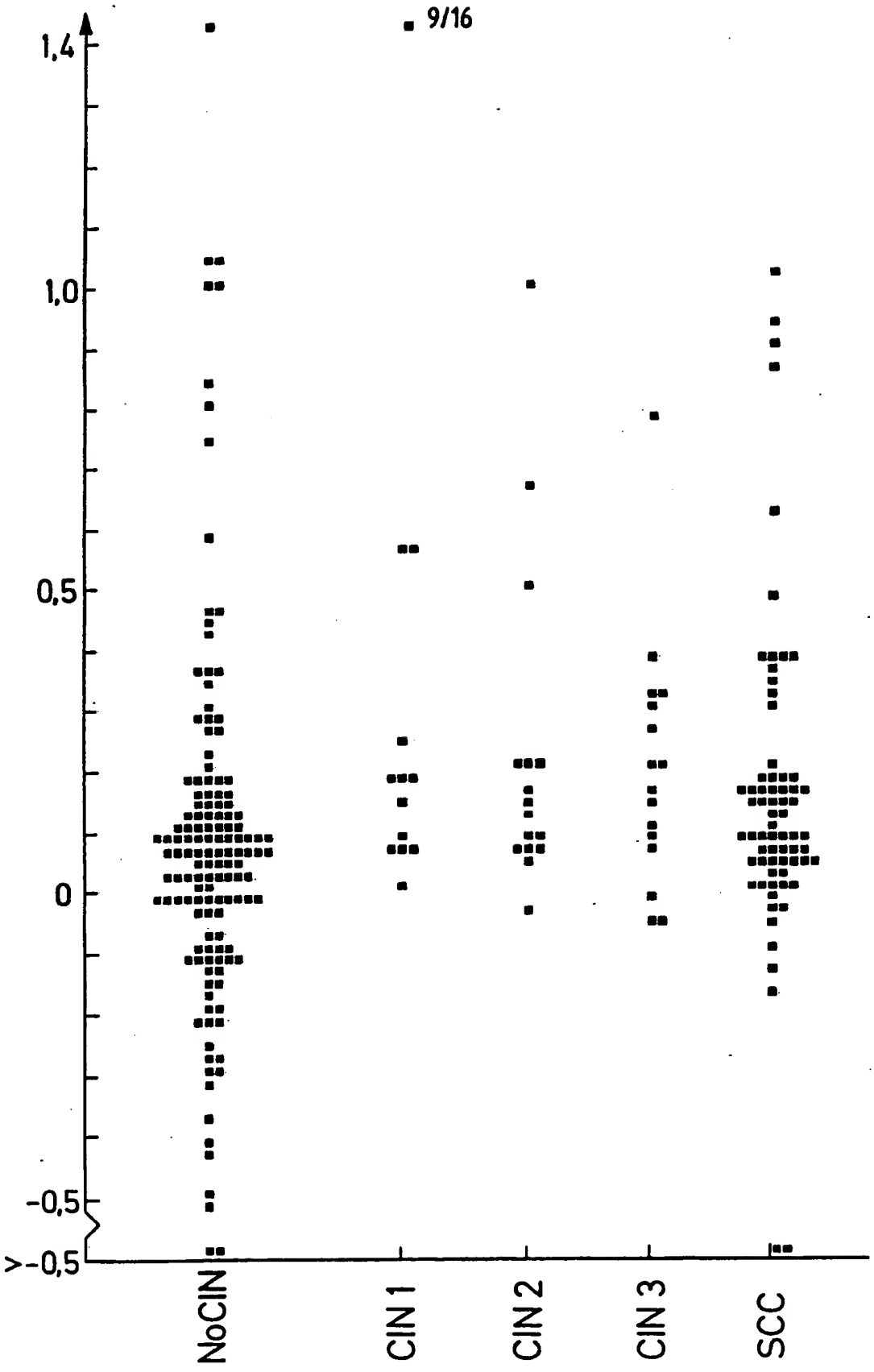


FIG. 11



Ig A - IgG, PV capsid antigen

FIG. 12

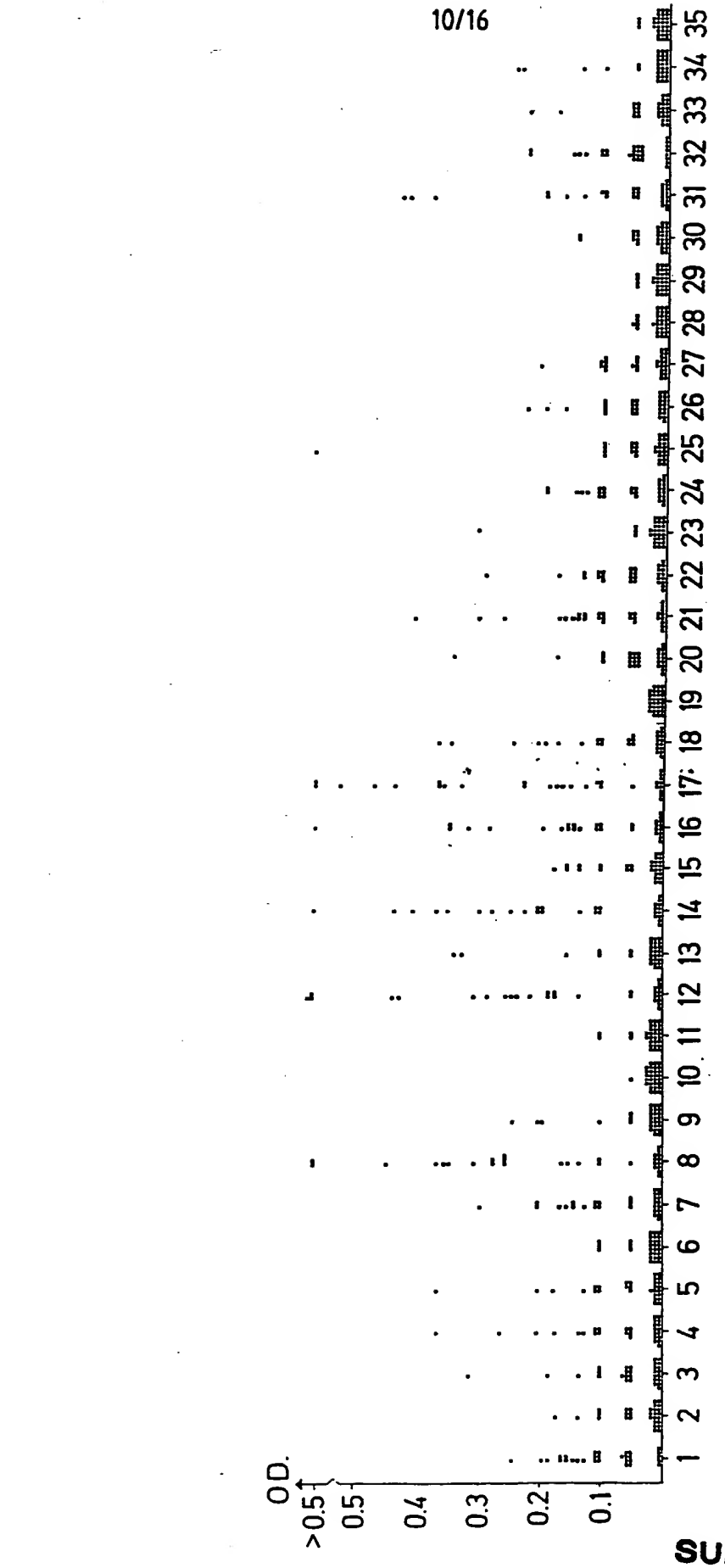
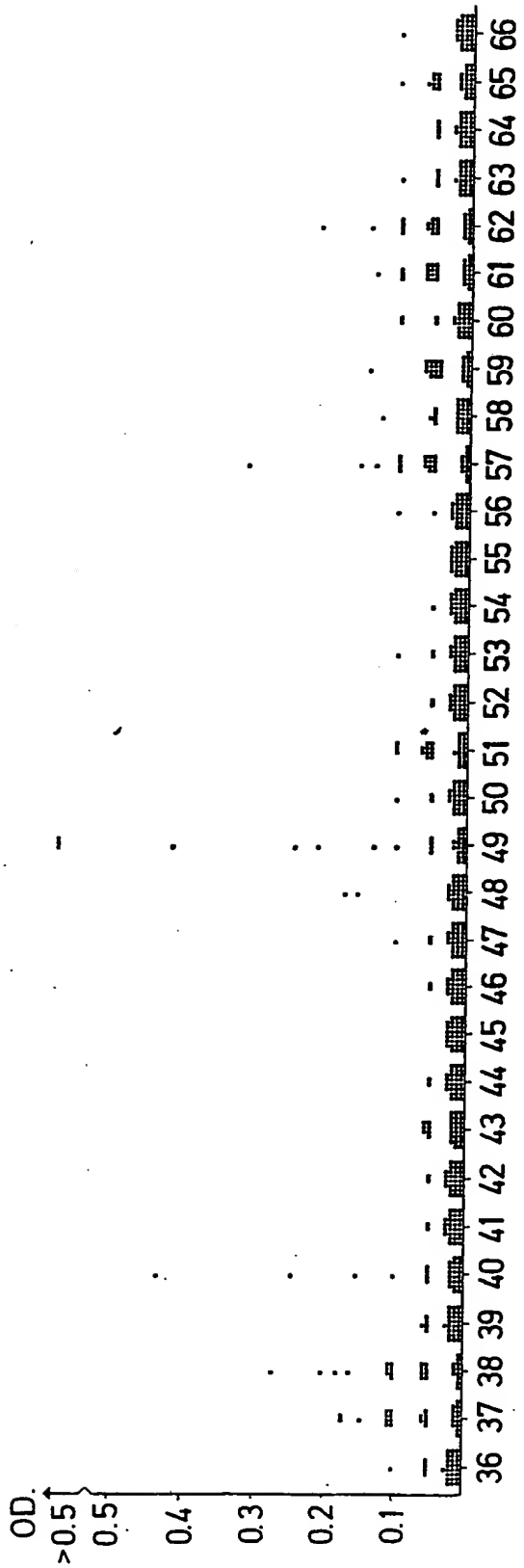

O.D.
>0.5
0.5
0.4
0.3
0.2
0.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

FIG. 13


OD.
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0.5
0.4
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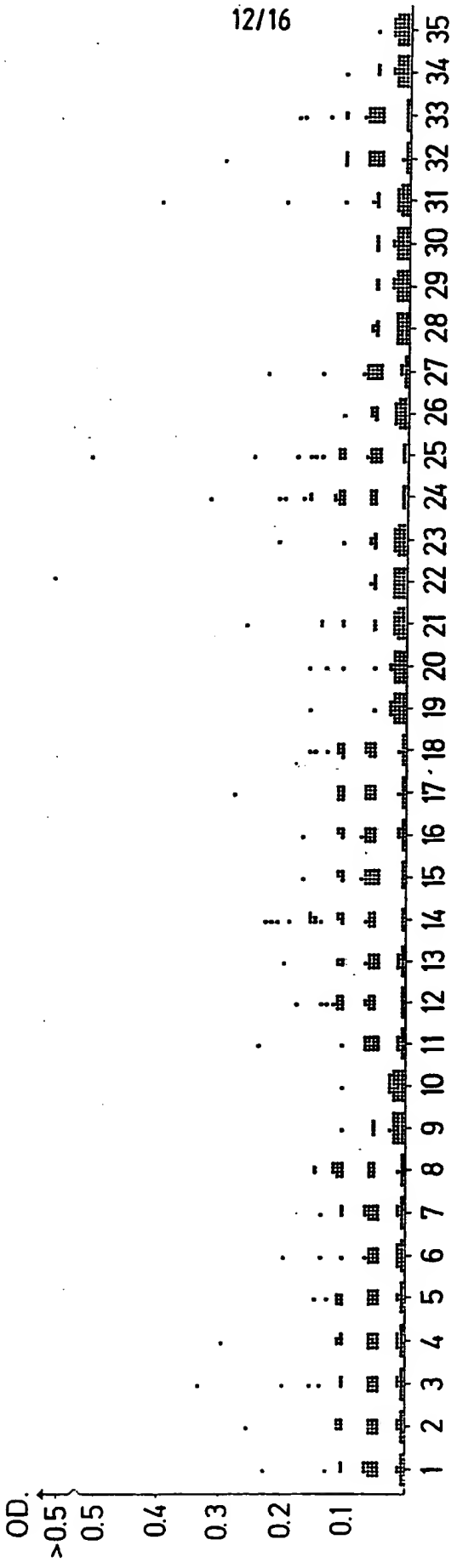
0.2
0.1
36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66

FIG. 14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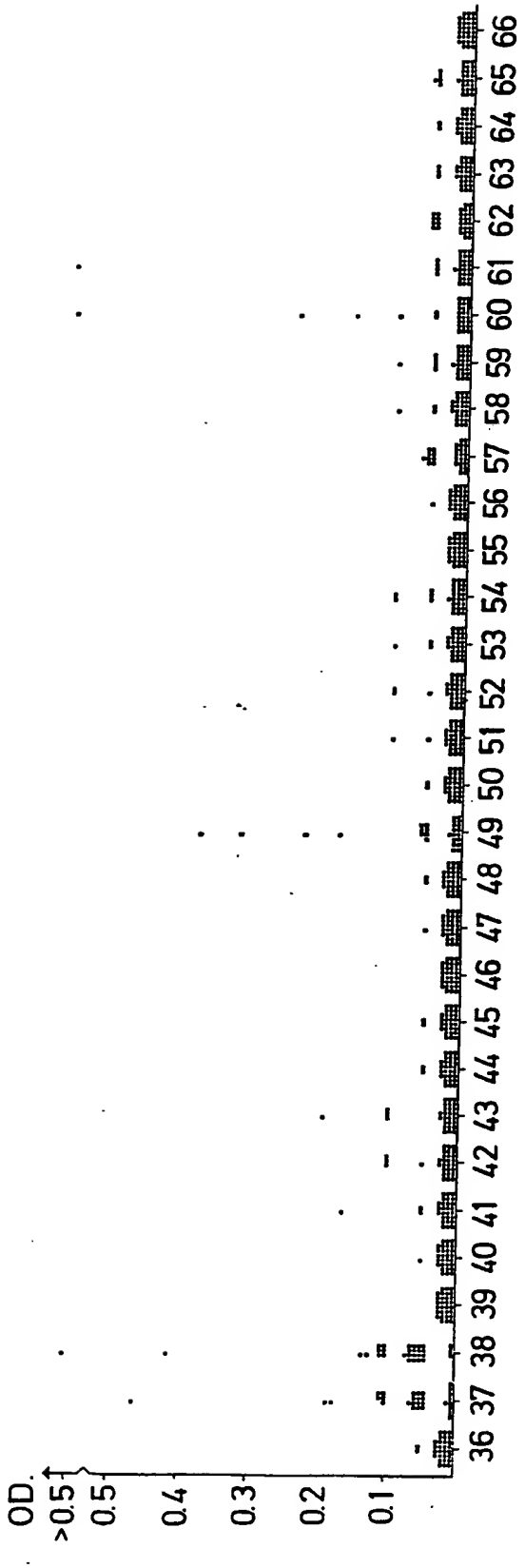
0.5
0.4
0.3
0.2
0.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

FIG. 15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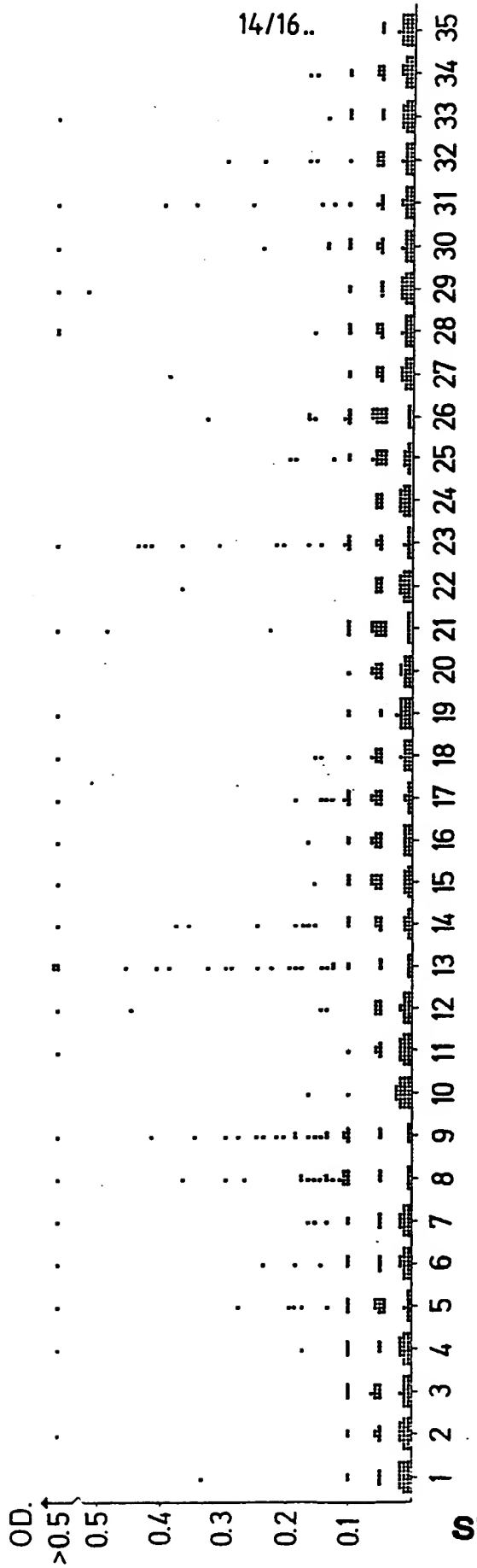
0.2
0.1
36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66

FIG. 16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0.1
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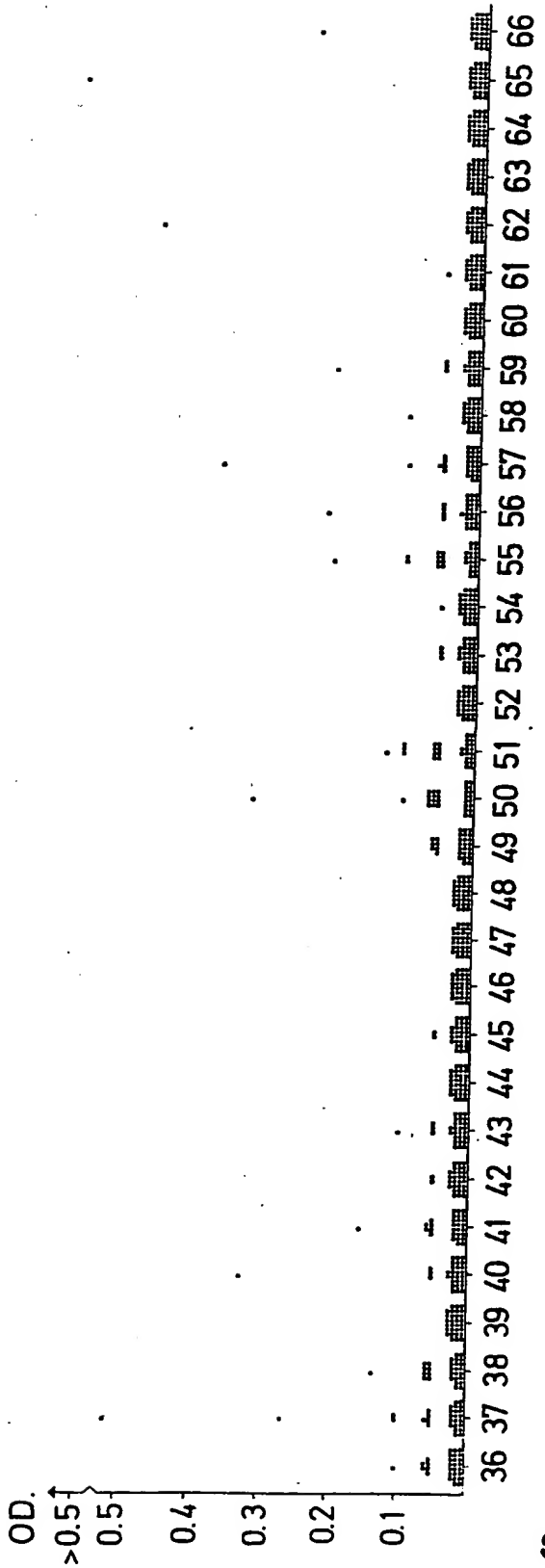
FIG. 17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0.4
0.3
0.2
0.1
36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66

FIG. 18

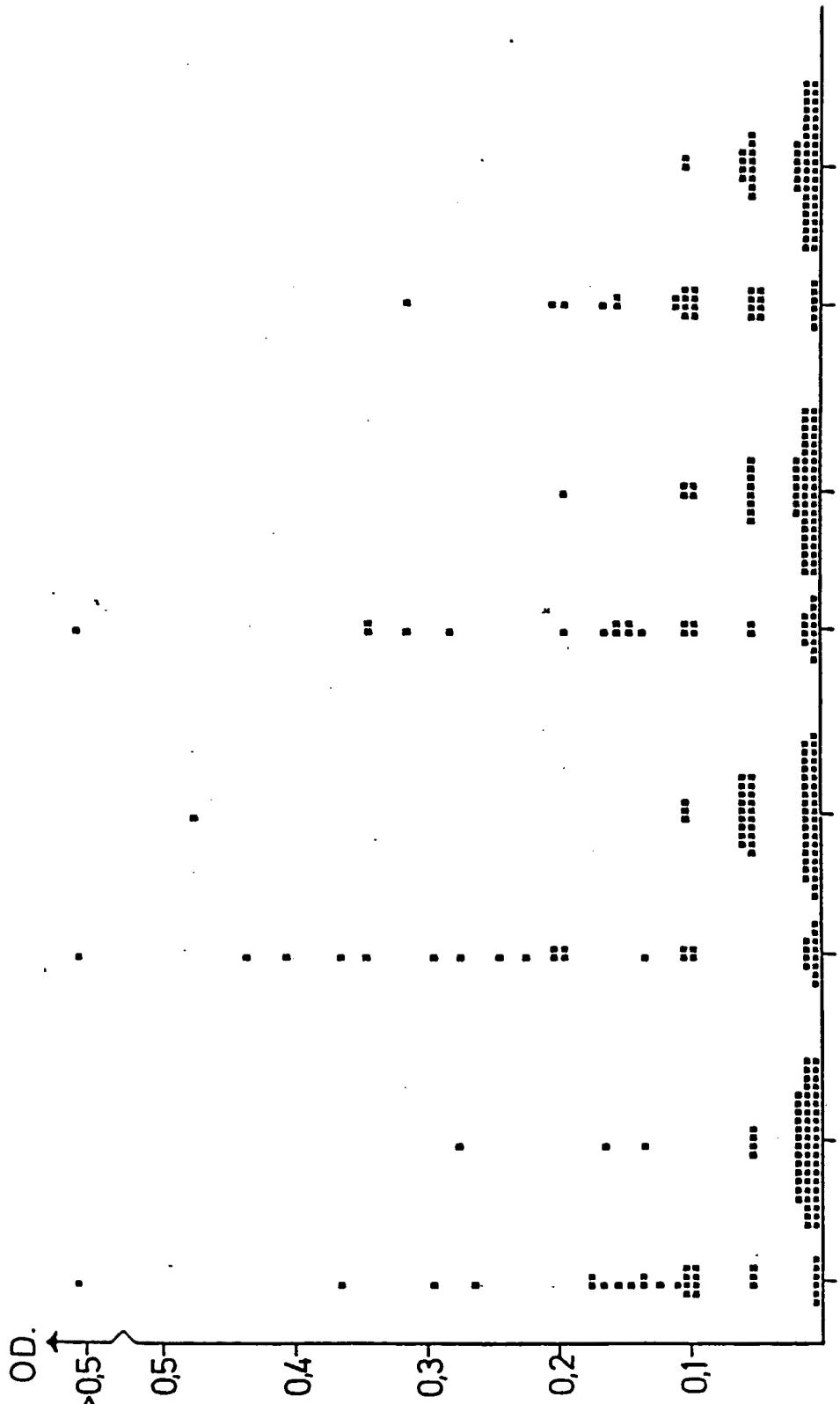
O.D.
>0,5
0,5
0,4
0,3
0,2
0,1
HPV 16 control SCC peptide 8, IgM
HPV 16 control SCC peptide 14, IgA
HPV 16 control SCC peptide 16, IgA
HPV 16 control SCC peptide 24, IgG

FIG. 19

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 89/00612

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: G 01 N 33/569, C 07 K 7/08

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	G 01 N; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	WO, A1, 87/01375 (INSTITUT PASTEUR, INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE) 12 March 1987, see the whole document --	1-40
X,Y	EP, A2, 0257754 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 2 March 1988, see the whole document and in particular the claims, pages 5-6 and fig. 5 and 6. --	1-40
X,Y	WO, A1, 86/05816 (GEORGETOWN UNIVERSITY) 9 October 1986, see in particular the claims and pages 13 and 26-30 --	1-40

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15th January 1990	1990 -01- 22
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carl Olof Gustafsson

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,Y	Chemical Abstracts, volume 107, no. 15, 12 October 1987, (Columbus, Ohio, US), Doorbar John et al: "Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus 1a. ", see page 395, abstract 130591g, & J. Virol. 1987, 61(9), 2793- 9	1-40
X,Y	Chemical Abstracts, volume 106, no. 17, 27 April 1987, (Columbus, Ohio, US), Seedorf K. et al: "Identification of early proteins of the human papilloma viruses type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. ", see page 152, abstract 132597s, & EMBO J. 1987, 6(1), 139- 44	1-40
A	EP, A1, 0243221 (INSTITUT PASTEUR) 28 October 1987, see pages 5-6 and 14-15 and claims 6 and 7	1,11,21, 31
Y	AU, B, 30071/84 (PHILLIP JOHN BAIRD) 3 January 1985, see in particular the claims and pages 12 and 7	1-40
Y	Dialog Information Services, Database Medline File 154, Dialog Accession no 06310140, Li C C et al: "Identification of the human papillomavirus type 6b L1 open reading frame protein in condylomas and corresponding antibodies in human sera", J VIROL Sep 1987, 61 (9) p. 2684-90.	1-40
A	National Library of Medicine database, Medline, File Med 83, NLM Accession no 85005364, Krisch I et al: "Demonstration of secretory component, IgA, and IgM by the peroxidase-antiperoxidase technique in inverted papillomas of the nasal cavities", & Hum Pathol 1984 Oct;15(10):914-20	1-40

FURTHER INFORMATION CONTINUED FR M THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

A posteriori non unity is deemed to create several independent inventions, eg. covering epitopes for IgA, IgG and/or IgM.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☒ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 89/00612

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 87/01375	12/03/87	FR-A-B- 2586428	27/02/87
		EP-A- 0235187	09/09/87
		JP-T- 63500662	10/03/88
EP-A2- 0257754	02/03/88	AU-D- 75535/87	14/01/88
		JP-A- 63183600	28/07/88
		US-A- 4777239	11/10/88
WO-A1- 86/05816	09/10/86	EP-A- 0217919	15/04/87
		JP-T- 62502378	17/09/87
EP-A1- 0243221	28/10/87	WO-A- 87/05630	24/09/87
		AU-D- 72007/87	09/10/87
		JP-T- 63502798	20/10/88
AU-B- 30071/84	03/01/85	JP-A- 60089755	20/05/85
		US-A- 4693199	15/09/87